MEETING

SCIENTIFIC ADVISORY BOARD

March 26, 27, 28, 1975

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MICROBIOLOGICAL ASSOCIATES

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OAK RIDGE

#15B - Machine Evaluation, Dosimetry and Smoke Fractionation

SCRIPPS

#16B - Immunologic Competence and Chemical Carcinogenesis

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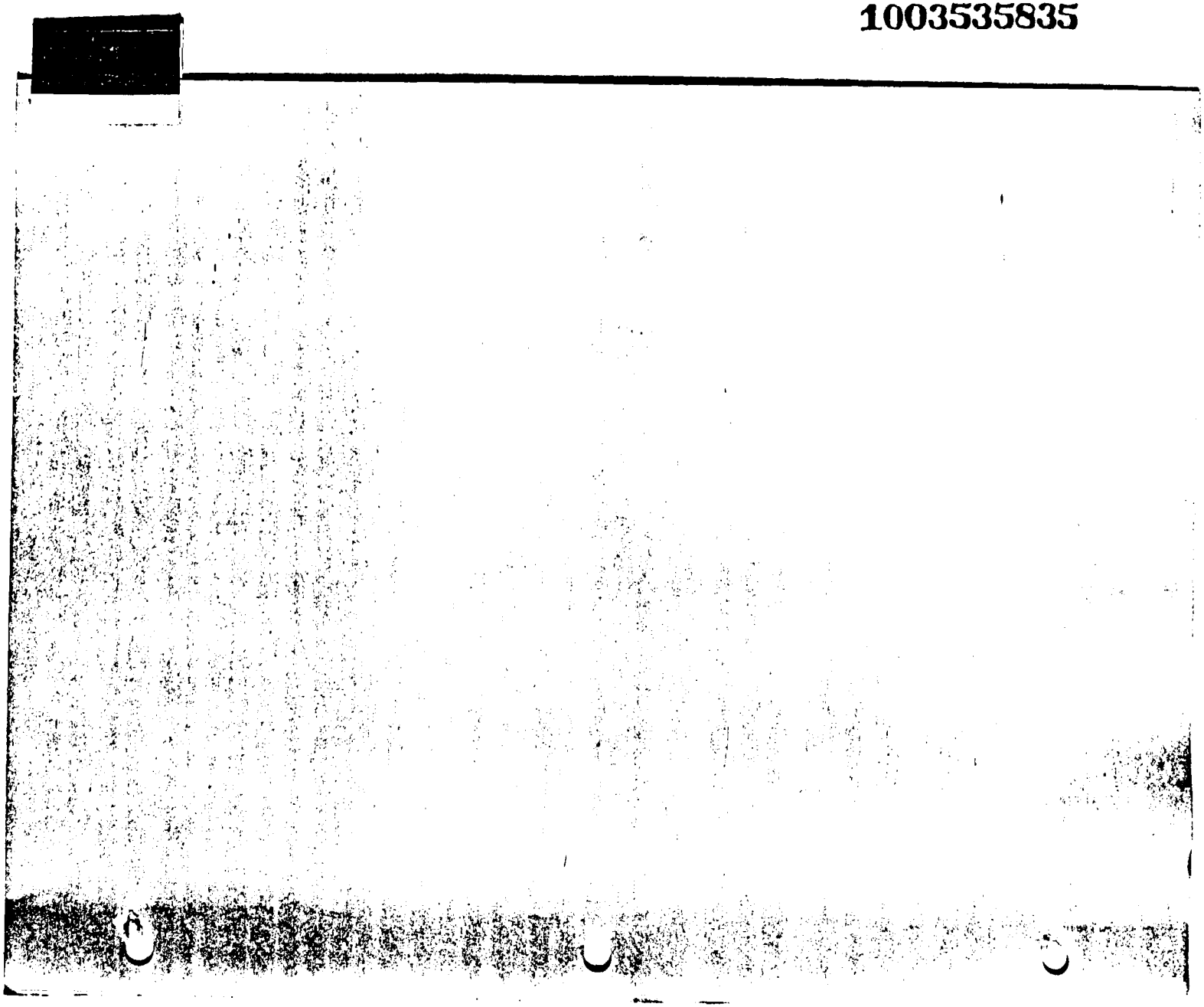

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Smoke Facility
and Inhalation

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SMOKE INHALATION CARCINOGENESIS STUDIES IN MICE

CTR Contract #22
MA Contract 2224

CONTRACT RENEWAL PROPOSAL
(SECOND YEAR)

FOR THE PERIOD

July 1, 1975 - June 30, 1976

1003535837

February 12, 1975

Vincent L. Ruwet
Vice President, Contracts and Administration

Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

TO: The Council for Tobacco Research U.S.A.
110 East 59th Street
New York, N.Y. 10022

FROM: Microbiological Associates
A Division of Dynasciences Corporation
4733 Bethesda Avenue
Bethesda, Maryland 20014

Prepared by:

Carrie E. Whitmire, Ph.D.
Project Director

10035838

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A. Smoke Inhalation Studies

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I Progress Report

Since this contract was initiated July 1, 1974 an architectural plan has been drawn, the building contract let and rearrangement of the facilities are well underway. We anticipate the building to be ready for occupancy by June 1, 1975. This will allow a month to prepare the facility for work by July 1, 1975 in the new contract year.

The equipment has been ordered and much of it has begun to arrive. The only item which has given us considerable problems has been the X-ray machine. We have not been able to achieve the desired resolution to provide for sensitive lung cancer diagnosis to date. There are several more approaches to be taken which may allow us to obtain this equipment in the new contract year.

The various studies to define the lung carcinogenesis bioassay system with BaP have been quite encouraging and have been reported on in the progress report for CTR-14.

The dosimetry studies with Oak Ridge National Laboratories has been initiated. A group of studies have been designed and are included in this proposal.

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II Proposal for New Contract Year

A. Smoke Studies

These were outlined in last year's proposal and will be more closely defined before initiated on occupation of the new facilities. At this time various experiments are still in progress which could influence the final experimental design.

B. Dosimetry Studies

Studies have been designed in collaboration with Dr. James Stockely at ORNL and are outlined on the following pages. These studies will be carried out on the Walton Smoking Machine. These data will be used to design dosimetry studies on the new smoking machine to be used in the inhalation studies.

1003535841

Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

PROTOCOL FOR MOUSE DOSIMETRY EXPERIMENTS ON WALTON HORIZONTAL SMOKING MACHINE

Prepared by

C.E. Whitmire
Microbiological Associates

and

J.R. Stokely
Oak Ridge National Laboratory

1003535842

Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

These protocols outline proposed collaborative studies by Microbiological Associates and Oak Ridge National Laboratory to investigate mouse dosimetry on the Walton Horizontal Smoking Machine. The objectives of these studies are:

1. To define the dose of tobacco smoke particulates received by mice under selected exposure conditions (exposure time and smoke concentration),

2. To ascertain possible effects of sex and strain on dosimetry so that a rational selection can be made for future studies of biological impact,

3. To determine retention and clearance rate of smoke particulates after exposure, and

4. To determine cumulative dose and long-term retention of smoke particulates under typical exposure regimes.

100353543

Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

Responsibilities of Oak Ridge National Laboratory are the following:

1. Weight and RTD selection of cigarettes.
2. Labeling of cigarettes with ^{14}C tracers.
3. Quality control of cigarettes (determination of nicotine, TPM, tar and ^{14}C delivery of selected radiolabeled cigarettes).
4. Shipping of radiolabeled cigarettes to Microbiological Associates.
5. Construction and testing of apparatus for sampling exposure chamber.
6. Instruction and assistance on operation of smoking machines, sampling apparatus, and cigarette conditioning and handling.
7. Analysis of animal tissues for ^{14}C tracers.
8. Analysis of input Cambridge filter pads for nicotine and ^{14}C tracer and chamber samples for ^{14}C tracer.
9. Calculations to obtain the following results:
   a. absolute activity (dpm) of tracer in each tissue specimen.
   b. absolute tar (mg) deposited in each tissue specimen based on tracer deposition.
   c. percent distribution of activity among various tissue specimens for each animal.
   d. dose expressed as percentage of smoke input to exposure chamber.

Responsibilities of Microbiological Associates are the following:

1. Calibration of smoking machines (puff volume, puff time, exposure time, purge time).
2. Conditioning and weighing of labeled cigarettes after receipt from Oak Ridge National Laboratory.
3. Collection of samples of smoke input to smoking machines and chamber samples obtained during animal exposures.
4. Animal conditioning.
5. Animal exposure.
6. Animal sacrifice and dissection.
7. Shipping of tissue specimens, input filter pads and chamber samples to Oak Ridge National Laboratory.

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Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

Experiment I. Effect of Exposure Time

1. One mouse strain: C3H/Anf Cum
2. One sex: female
3. Four exposure time intervals: 10,20,30,40 seconds
4. One exposure concentration: 10%
5. Four tissue samples: skinned head, upper trachea and larynx, lungs and lower half of trachea, stomach and esophagus
6. Number of mice per exposure: 10(plus 10 scrubs)
7. Number of cigarettes per exposure: 3 (2 for chamber input, 1 for exposure)
8. Chamber samples per exposure: 1
9. Cigarette type: Kentucky Reference 1A1 loaded with 5×10^6 dpm ^{14}C-dotriacontane--weight and RTD tested ($\pm$ 20 mg, $\pm$ 5 mm H_2O)
10. Number of repetitive exposures: 4 (40 Mice)
11. Instant sacrifice
12. Tissue specimens: 640
13. Total cigarettes: 60 (assume 25% loss).

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Experiment II. Effect of Smoke Concentration

1. One exposure time interval: based on experiment I
2. Five exposure concentrations: 20% (2 cigarettes-384 ml chamber),
   30% (3 cigarettes-384 ml chamber),
   5% (1 cigarette-768 ml chamber),
   10% (2 cigarettes-768 ml chamber),
   15% (3 cigarettes-768 ml chamber)
3. Number of cigarettes per exposure: 6(20%), 9(30%), 3(5%), 6(10%), 9(15%)
4. One mouse strain: C3H/Anf Cum
5. One sex: female
6. Four tissue samples: skinned head, upper trachea and larynx, lungs and lower half of trachea, stomach and esophagus
7. Number of mice per exposure: 10 (plus 10 scrubs)
8. Chamber samples per exposure: 1
9. Cigarette type: Kentucky Reference 1A1 loaded with 5×10^6 dpm ^{14}C-dotriacontane--weight and RTD tested ($\pm$ 20 mg, $\pm$ 5 mm H_2O)
10. Number of repetitive exposures: 4 (40 mice)
11. Instant sacrifice
12. Total tissue specimens: 800
13. Total cigarettes: 165 (assume 25% loss)

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Experiment III. Effect of Sex

1. Two sex: male and female
2. One exposure time interval: based on experiment I
3. One exposure concentration: based on experiment II
4. Number of mice per exposure: 20 (10 male, 10 female)
5. Number of repetitive exposures: 4 (40 mice)
6. Four tissue samples: skinned head, upper trachea and larynx, lungs and lower half of trachea, stomach and esophagus
7. Chamber samples per exposure: 1
8. Cigarette type: Kentucky Reference 1A1 loaded with 5×10^6 dpm ^{14}C-dotriacontane--weight and RTD tested ($\pm$ 20 mg, $\pm$ 5 mm H_2O)
9. Instant sacrifice
10. Tissue specimens: 300
11. Total cigarettes: 15 (assume 25% loss).

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Experiment IV: Strain Differences

1. Four mouse strains: C3H/f, C57BL/6, DBA/2, B6C3F1
2. Sex: based on experiment III (male, female, both)
3. One exposure time: based on experiment I
4. One exposure concentration: based on experiment II
5. Four tissue samples: skinned head, upper trachea and larynx, lungs and lower half of trachea, stomach and esophagus
6. Number of mice per exposure: 20 (10 each of 2 strains or sexes
7. Number of cigarettes per exposure: 3 (2 for chamber input, 1 for exposure)
8. Chamber samples per exposure: 1
9. Cigarette type: Kentucky Reference 1A1 loaded with 5×10^6 dpm ^{14}C-dotriacontane--weight and RTD tested (±20 mg, ± 5 mm H_2O)
10. Instant sacrifice
11. Tissue specimens: 640 (1 sex)
    1280 (2 sexes)
12. Number of cigarettes: 30 (1 sex)
    60 (2 sexes) (assume 25% loss)

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Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

Experiment V: Retention Period of ^{14}C-Dotriacontane

1. One mouse strain: C3H/f
2. One sex: based on experiments III and IV
3. One exposure time: based on experiment I
4. One exposure concentration: based on experiment II
5. Five tissue samples per animal: same as experiment I plus composite sample of all other animal tissues--animal skinned--skin not included in composite.
6. Number of mice per exposure: 20
7. Five sacrifice times after smoking: 0.25, 1, 4, 16, 24 hours. Four animals sacrificed at each time.
8. Number of cigarettes per exposure: 3
9. Cigarette type: 1A1 loaded with maximum amount (up to 1×10^8 dpm) ^{14}C-ditriacontane.
10. Number of repetitive exposures: 4
11. Tissue specimens: 400
12. Number of cigarettes: 15 (assume 25% loss)

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Experiment VI: Retention Period of ^{14}C-Benz(a)pyrene

1. Cigarette type: 1A1 loaded with maximum amount (up to 1 x 10^8 dpm) ^{14}C-benz(a)pyrene
2. One mouse strain: C3H/f or B6C3F1
3. One sex: based on experiments III and IV
4. One exposure time: based on experiment I
5. One exposure concentration: based on experiment II
6. Five tissue samples per animal: same as experiment I plus composite sample of all other animal tissues--animal skinned--skin not included in composite.
7. Number of mice per exposure: 20
8. Five sacrifice times after smoking: 0.25, 1, 4, 16, 24 hours. Four animals sacrificed at each time.
9. Number of cigarettes per exposure: 3
10. Cigarette type: 1A1 loaded with maximum amount (up to 1 x 10^8 dpm) ^{14}C-ditriacontane.
11. Number of repetitive exposures: 4
12. Tissue specimens: 400
13. Number of cigarettes: 15 (assume 25% loss)

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Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

Experiment VII: Retention Period of ^{14}C-Nicotine

1. Five sacrifice times after smoking: immediately, 0.25, 0.5, 1, 4 hours after smoking. Four animals sacrificed at each time.
2. Cigarette type: 1A1 loaded with maximum amount (up to 1×10^8) ^{14}C-nicotine.
3. One mouse strain: C3H/f
4. One sex: based on experiments III and IV
5. One exposure time: based on experiment I
6. One exposure concentration: based on experiment II
7. Five tissue samples per animal: same as experiment I plus composite sample of all other animal tissues--animal skinned--skin not included in composite.
8. Number of mice per exposure: 20
9. Five sacrifice times after smoking: 0.25, 1, 4, 16, 24 hours. Four animals sacrificed at each time.
10. Number of cigarettes per exposure: 3
11. Cigarette type: 1A1 loaded with maximum amount (up to 1×10^8 dpm) ^{14}C-dotriacontane.
12. Number of repetitive exposures: 4
13. Number of cigarettes: 15 (assume 25% loss).

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Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

Experiment VIII: Comparative Retention Periods for Two Mouse Strains

1. Two strains: based on experiment IV
2. One sex: based on experiments III and IV
3. One exposure time: based on experiment I
4. One exposure concentration: based on experiment II
5. Two retention periods: based on experiments V, VI, and VII
6. Five tissue specimens per animal: experiment V
7. Twenty mice per exposure: ten mice sacrificed at each retention period
8. Number of repetitive exposures: 2
9. Three type cigarettes: Kentucky Reference 1A1 loaded with ^{14}C-nicotine, ^{14}C-benz(a)pyrene, or ^{14}C-dotriacontane (maximum load)
10. Tissue specimens: 1200
11. Number of cigarettes: 15(assume 25% loss).

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Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

Experiment IX: Cumulative Dose and Long Term Retention

1. Number of radiolabeled cigarettes given per 8 hour day: 2, 5, 10, 20
2. One strain - based on experiment VIII
3. Four animals sacrificed at each of the following times after exposure: immediately, 4, 24, 48, 120 hours
4. Three type cigarettes: Kentucky Reference 1A1 loaded with ^{14}C-nicotine, ^{14}C-benz(a)pyrene, or ^{14}C-dotriacontane (maximum load) or all three at one time.
5. Number of repetitive tests: 1
6. Other conditions: same as experiment VIII
7. Tissue specimens: 1200
8. Number of cigarettes: 169 (assume 25% loss).

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Summary of Tissue Specimens and Number of Cigarettes

Experiment	Tissue Specimens[1]	Number of Cigarettes[2]
I	640	60
II	800	165
III	320	15
IV	1280	60
V	400	15
VI	400	15
VII	400	15
VIII	1200	15
IX	1200	169
	6640	529

[1] Maximum

[2] Assume 25% loss

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Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

III Budget

The budget has been divided into the various support and scientific sections required to carry out the proposed studies. Several items of equipment have been included in this year's budget which will be needed to carry out the program.

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BUDGET CTR-22 (July 1, 1975 - June 30, 1976)

		Section A*	B	C	D	E	Totals
A.	Direct Labor (Schedule A)	$ 23,240	$ 20,234	$ 29,573	$ 54,509	$110,645	$238,201
B.	Overhead (115% of A)	26,726	23,269	34,009	62,685	127,242	273,931
C.	Other Direct Costs	--	7,000	35,000	10,000	45,000	97,000
D.	Travel	2,000	--	--	1,500	--	3,500
E.	Total (A-D)	51,966	50,503	98,582	128,694	282,887	612,632
F.	G & A (16% of E)	8,315	8,080	15,773	20,591	45,262	98,021
G.	Total	60,281	58,583	114,355	149,285	328,149	710,653
H.	Fixed Fee	6,698	6,509	12,705	16,586	36,457	78,955
I.	Total Before Equipment and Overtime Premium	66,979	65,092	127,060	165,871	364,606	789,608
J.	Equipment	1,500	300	8,000	5,500	10,000	25,300
K.	Overtime Premium	--	--	--	1,500	--	1,500
L.	Total	$ 68,479	$ 65,392	$135,060	$172,871	$374,606	$816,408
M.	Contingency						83,592
N.	Total Cost						$900,000

*Sections: A. Administration
B. Data Processing
C. Dosimetry with ORNL
D. Histopathology
E. Inhalation Studies

100353856

Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

SCHEDULE A - Direct Labor For CTR Contract 22
(July 1, 1975 - June 30, 1976)

Section/Personnel	$/Hr.	% Time	Hours	$
A. Administration				
C.E. Whitmire, Ph.D.	14.38	50	963	$13,848
C.F. Demoise, Ph.D.	8.18	25	482	3,943
Vacancy, Adm. Assist.	4.08	50	963	3,929
			2,408	$21,720
		7% Merit Raise		1,520
				$23,240
B. Data Processing				
M. Haven, M.S.	10.58	50	963	$10,189
P. Gradwell, Data Tech.	3.84	30	578	2,220
Vacancy, Data Tech.	3.50	50	963	3,371
B. Ross, Key Punch Tech.	3.25	50	963	3,130
			3,467	$18,910
		7% Merit Raise		1,324
				$20,234
C. Dosimetry Studies with ORNL				
D. Dansie, Tech.	3.80	100	1,926	$ 7,319
Vacancy, Tech.	4.20	100	1,926	8,089
G. Gomez, Jr. Tech.	3.25	100	1,926	6,259
J. Fernandez, Anim. Care.	3.10	100	1,926	5,971
			7,704	27,638
		7% Merit Raise		1,935
				$29,573
D. Histopathology				
Vacancy, D.V.M.	17.30	100	1,926	$33,320
S.A. Gosnell, Super. Tech.	4.30	50	963	4,141
Vacancy, Tech.	3.50	100	1,926	6,741
Vacancy, Tech.	3.50	100	1,926	6,741
			6,741	$50,943
		7% Merit Raise		3,566
				$54,509
E. Inhalation Studies				
7 Vacancies, Jr. Tech.	3.50	100	13,482	$47,187
4 Vacancies, Jr. Tech.	3.50	50	3,852	13,482
A. Saborit, Washroom Aide	3.39	100	1,926	6,529
2 Vacancies, Washroom Aide	3.20	100	3,852	12,326
1 Vacancy, Anim. Care.	3.35	100	1,926	6,452
A. Lopez, Sr. Tech.	4.80	100	1,926	9,245
G. Griffin, Tech.	4.25	100	1,926	8,18[illegible]
			28,890	$103,407
		7% Merit Raise		7,238
				$110,645
Totals	25.5 Man Years		49,210	$238,201

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Schedule B: CTR-22 (July 1, 1975 - June 30, 1975)

Desks, files	$ 450
Typewriter	650
2 calculators	400
Computer files	300
Gas chromatographic and other dosimetry monitoring equipment	8,000
Pathologist's microscope tables, etc.	1,500
Microprojector	4,000
X-ray machine and safety shield	10,000
	$25,300

10035358

Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

Basic and
Supportive Research

1003535859

Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

March 4, 1975

To: The Scientific Advisory Board

Subject: Basic and Supportive Contract No. 14B - Microbiological Associates, July 1, 1975 - June 30, 1976

The studies to determine susceptibility to lung carcinogenesis in genetically defined mouse strains have been carried out under the direction of Drs. Carrie Whitmire and Richard Kouri at Microbiological Associates.

The activities to be undertaken during each funding period are proposed by the contractor with close staff liaison. The Scientific Director and Research Director further refine these proposals, deleting or adding as felt appropriate. Site visits are arranged between contractor, SAB members of the contract committee, CTR representatives including Scientific Director and Research Director, and, as of the forthcoming period of Contract No. 14B, outside consultants with special scientific expertise in the area under consideration. (Periodic site visits are made during the year to follow up the progress). Recommendations and proposals are then submitted to the SAB for formal considerations. Final budgetary negotiations are carried out by CTR's Scientific Director and contractor following the SAB meeting and in line with budgetary recommendations, limitations, etc. of the CTR.

The current proposal, subject to negotiations, revision pending, recommendations of reviewing groups, etc. is attached. This represents a consolidation of Grants No. 2 and 14, arbitrarily divided some years ago for budgetary considerations.

Funding history for the past year:

		Amount	Annual Amount
No. 2F	2/1/74 - 1/31/75	$137,500	
No. 14A	7/1/74 - 6/30/75	$350,000	
			$487,500
No. 14AS	Supplement 1/31/75 - 6/30/75 (to bring two contracts to same initiation date).	$ 58,000	
Requested (subject to SAB approval, negotiation, etc.)			
No. 14B	7/1/75 - 6/30/76		$522,794

J.H.K.

1003535860

Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

Experimental Lung Cancer

Carcinogenesis and Bioassays

International Symposium
Held at the Battelle Seattle Research Center
Seattle, WA, USA, June 23-26, 1974

Edited by
Eberhard Karbe and James F. Park

With 312 Figures and 144 Tables

Springer-Verlag Berlin Heidelberg New York 1974

100353928

Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

The Significance of Aryl Hydrocarbon Hydroxylase Enzyme Systems in the Selection of Model Systems for Respiratory Carcinogenesis*

Richard E. Kouri, Charles F. Demoise, and Carrie E. Whitmire

Department of Experimental Oncology, Viral-Chemical Carcinogenesis Section, Bethesda, MD 20014, USA

ABSTRACT

Aryl hydrocarbon hydroxylase (AHH) is a multicomponent, microsomal-bound enzyme system which converts a variety of lipid-soluble compounds to water-soluble forms for subsequent elimination from the body. The enzyme system is inducible by a variety of endogenous and exogenous compounds including steroid hormones, barbiturates, insecticides, polycyclic aromatic hydrocarbons (PAH), and whole cigarette smoke. Recent results have demonstrated that inducibility is host-gene regulated, the inducibility of this enzyme correlates with carcinogenic susceptibility to PAH in animal, and bronchogenic squamous cell carcinoma in humans (probably cigarette smoke induced).

This paper illustrates the types of AHH responses observed in pulmonary tissues following treatment of mice of various strains with either PAH or tobacco related chemicals. Following intratracheal instillation of 3-methylcholanthrene, we observed that: a) pulmonary AHH can be induced preferentially at doses <200 µg (in contrast to higher doses that induce both hepatic and pulmonary tissues), b) kinetic data demonstrate a 6 to 8 fold increase within 24 hours followed by a broad plateau lasting up to 96 hours, and c) induction is host regulated, segregating as a single autosomal dominant gene in crosses between the C57BL/6 (inducible) and DBA/2 (noninducible) strains of mice. Although DBA/2 pulmonary tissue is slightly inducible (in contrast to the noninducibility of hepatic tissue), evidence indicates that this response results from proliferation of constitutive AHH and not true "induction". Exposure to whole smoke from one 1A1 cigarette (10% smoke in a Walton Horizontal Smoking Machine) will preferentially induce pulmonary AHH, and this response is under the same genetic control as that induced by MCA. Exposure to gas phase alone will not induce this response. Use of cigarette smoke condensate fractions (Stedman fractionation) derived from 1A1 tobacco show that after intratracheal instillation, at least 4 fractions are capable of inducing pulmonary AHH. Fractions 3, 4, 12, and 14 (Bi^a, Bi^b, N_{NM}, and N_{MEOH}) induce at least a 2 fold increase of pulmonary AHH (at a LD_{10-14} dose). It seems as if the enzymatic potential of the lung tissue itself may be a major determinant in the ultimate fate of this organ in any carcinogenic process.

*This research was supported by the Council for Tobacco Research.

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Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

A. Introduction

Aryl hydrocarbon hydroxylase (AHH) is the name given to one of the multi-component, mixed-function oxidases that converts a variety of lipid-soluble endogenous and exogenous compounds to water-soluble forms, usually for subsequent elimination from the body (MASON, 1957; CONNEY, 1967). The enzyme system possesses 2 properties which make it particularly amenable for studying its role in chemically induced cancers: 1. the system is inducible**(NEBERT and GELBOIN, 1969) and 2. this inducibility is regulated by a single autosomal dominant gene in crosses involving the C57BL/6 and DBA/2 strains of mice (THOMAS et al., 1972; NEBERT et al., 1972). Recent information suggests that this enzyme system plays a major role in 3-methylcholanthrene-induced carcinogenesis in the aforementioned mouse strains (KOURI et al., 1973a, 1973b, 1974a).

The lung and skin of mice seem to be under a different type of genetic control from that of hepatic tissue, because these organs appear to be slightly inducible in strains in which the liver is completely non-inducible (BURKI et al., 1973; WIEBEL et al., 1973). There are many questions concerning the pulmonary response: Is there really a separate genetic control for lung AHH levels? Do genetically regulated differences in AHH inducibility exist in pulmonary tissue of inbred mice? What is the effect of other exogenous chemicals (e.g., tobacco-related products) on lung AHH? Do these enzymatic responses play a role in the susceptibility of mice to chemically induced lung cancers? In this report, we attempt to answer some of the questions concerning the pulmonary response.

B. Materials

The polycyclic hydrocarbons benzo(a)pyrene (BaP) and 3-methylcholanthrene (MCA) were purchased (Sigma Chemicals, St. Louis, Missouri) and purified by recrystallization from benzene. 7,8-benzoflavone was purchased from Aldrich Chemicals (Cedar Knolls, New Jersey). Sources of mice were Cumberland View Farms (Clinton, Tennessee), the Jackson Laboratory (Bar Harbor, Maine), or Microbiological Associates (Bethesda, Maryland). For intratracheal (IT) instillations, a Bausch and Lomb stereomicroscope, equipped with fiber optic illumination, and Hamilton syringes with 22 gauge by 38 mm (with 1.5 mm feeder balls) needles were used. Fractions of cigarette smoke condensate from 1A1 cigarettes were provided by Dr. A.R. PATEL (Meloy Laboratories, Springfield, Virginia)(PATEL et al., 1974). Fractionation into acidic, basic, and neutral fractions was performed according to the procedures of SWAIN et al. (1969). Only 3 fractions have been analyzed as to chemical content: the B_E fraction contains 310 mg nicotine per g fraction, the WA_E fraction contains 41.9 mg phenols per g fraction, and the N_{NM} fraction contains 13.2 µg BP per g fraction (PATEL et al., 1974). Walton-type horizontal smoking machines were obtained from Process and Instruments (Brooklyn, New York), cigarettes were either the 1A1 or 1R1 type (University of Kentucky, Lexington). Enzyme determinations were made

** The term "inducibility", as used in this paper, denotes a relative increase in rates of de novo synthesis or of activation of enzyme activity from preexisting moieties, or in rate of both when compared to rate of breakdown. No particular mechanism is implied.

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Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

using an Aminco-Bowman spectrophotofluorometer (American Instrument Company, Silver Spring, Maryland).

C. Methods

Care and feeding of mice were as previously published (WHITMIRE et al., 1971). Animals were always treated between the hours of 9:00 am and 10:00 am to avoid diurnal variations. The intratracheal instillation technique was similar to that described recently by HO and FURST (1973). Solutions consisted of MCA suspended in 0.2% gelatin in sterile saline or the various cigarette smoke condensate fractions dissolved in corn oil. Condensate fractions were used at an arbitrary level that killed 10% of the mice in 14 days (LD_{10-14}). 0.02 ml of solution was instilled. At various times post-treatment, lungs and livers were excized and frozen at -70°C until assayed.

Microsomes were prepared from liver tissues of mice pretreated with MCA according to the methods of KUPFER and LEVIN (1972) and were stored at -70°C for up to 72 hours before being assayed. Calcium-aggregated and "normal" centrifugally prepared microsomes were used with similar results. Samples were diluted with 0.1M tris-HCl buffer (pH 7.4) to a final ratio of 1.0 ml microsome suspension per wet weight tissue.

The assay for AHH was basically that of NEBERT and GELBOIN (1969), as modified by NEBERT and GIELEN (1972) and THOMAS et al. (1972). A unit of AHH activity is that amount of enzyme causing the fluorescent equivalent of 1.0nMole 3-OHBP per min at 37°C. For hepatic and pulmonary tissues, activity is given in terms of units/g wet weight tissue and for microsome preparations in terms of units/mg protein.

Inhibition of BaP metabolism *in vitro* was done according to the procedures of GOUJON et al. (1972) and WIEBEL et al. (1971). Concentrations of the various condensate fractions were made in dimethylsulfoxide (DMSO); included was the known competitive inhibitor of "induced" AHH activity, 7,8-benzoflavone (WIEBEL et al., 1971). 200 µg, 20 µg, and 2 µg of the fractions were added to the complete reaction mixture (minus BaP), incubated with shaking for 1.0 min, and then BaP (20 µg) was added and incubation continued for 20 min.

Mice were exposed to 1, 2, or 3 cigarettes simultaneously (10%, 20%, or 30% smoke) and were also exposed to the smoke of 1 cigarette (10% smoke) for 1.0 hour (about 7 cigarettes). This latter exposure regimen consisted of exposing mice to 1 cigarette followed by a 10 min rest period followed by 1 cigarette, until a total of 7 cigarettes were smoked. In some cases, mice were exposed to cigarette smoke for at least 60 days at 8 cigarettes per day (4 consecutive cigarettes in morning and 4 in afternoon). At various times after exposure, mice were killed by cervical dislocation and the lungs and livers were removed and stored at -70°C until assayed. Control animals consisted of either untreated, sham-smoked, or gas-phase (Cambridge-filtered smoke) treated animals. Mice were exposed in 1 min cycles consisting of a 2 sec puff, 15 sec holding time, and 43 sec purge. In certain experiments, holding time was increased to 28 sec and purge time was 30 sec.

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D. Results

I. Pulmonary and Hepatic AHH Responses to IT Instilled MCA

The pulmonary and hepatic AHH levels 24 hours after IT administration of various dose of MCA into C57BL/6Cum (B6) DBA/2Cum (D2) and B6D2F1 mice are shown in Table 1. At doses greater than 200 µg, the AHH responses of pulmonary tissues from B6 and B6D2F1 lungs were maximally induced (about 7 fold) while hepatic tissues were only minimally effected. The D2 strain was generally much less responsive: pulmonary tissue was only minimally induced at a dose of 500 µg and hepatic tissue was never induced, regardless of MCA dose. Kinetics of induction of pulmonary AHH in these 3 strains following IT treatment with 200 µg MCA are shown in Fig. 1. Maximum induction in the B6 and B6D2F1 strains occurred by 24 hours and remained constant for at least 96 hours. The D2 strain was observed to respond slowly to MCA and maximal induction occurred 48 hours posttreatment. The maximum observed increase (inducibility) for the B6 or B6D2F1 strains was about 10 and for the D2 strain about 6.

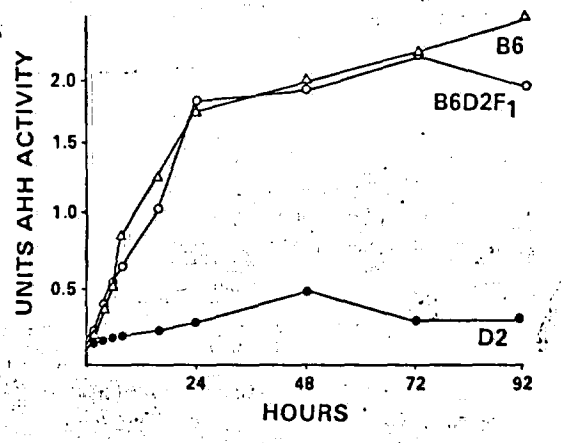

Fig. 1

Responses of 8 other inbred strains to 200 µg MCA given IT are shown in Table 2. Pulmonary tissues of BALB/cMai C3H/fMai, C57L/J, and C57BL/6J were observed to be highly induced by 200 µg MCA (4 to 8 fold), while lung tissue from strains AKR/J, SJL/J, DBA/2J, and RF/J showed no such increase. Hepatic responses were low for all strains except perhaps for the C57BL/6J and C57L/J, which did express a 1-fold increase..

II. Genetic Regulation of Pulmonary AHH Induction

The effect of MCA on pulmonary tissue from crosses involving the B6 and D2 strains is shown in Table 3. Animals were classified as inducible or noninducible if, after IT treatment with 200 µg MCA, pulmonary AHH levels were 2.5 (± 0.3) units/g tissue (inducible) or 0.3 (± 0.05) units/g tissue (noninducible). Among 47 backcross animals

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Table 1. Effects of intratracheal instillation of various doses of MCA in a 0.2% gelatin solution on pulmonary and hepatic AHH[a] levels

STRAIN AND TISSUE	UNTREATED	GEL	MCA 10 µg	MCA 50 µg	MCA 200 µg	MCA 500 µg
C57BL/6Cum						
LUNG	0.40	0.32	0.89 (2.8)[b]	1.4 (4.4)	2.4 (7.5)	2.4 (7.5)
LIVER	17.50	14.50	13.30 (0.9)	14.51 (1.0)	32.3 (2.2)	33.2 (2.3)
DBA/2Cum						
LUNG	0.30	0.18	--	0.20 (1.1)	0.34 (1.9)	0.56 (3.1)
LIVER	9.80	9.50	--	9.80 (1.0)	10.10 (1.1)	10.20 (1.1)
B6D2F1Cum						
LUNG	0.36	0.26	0.76 (2.9)	1.3 (5.0)	2.0 (7.7)	2.5 (9.6)
LIVER	12.60	10.00	10.30 (1.0)	14.4 (1.4)	18.9 (1.9)	26.5 (2.7)

[a]AHH ACTIVITY GIVEN IN TERMS OF UNITS PER g WET WEIGHT TISSUE. A UNIT IS THAT AMOUNT OF ENZYME CAUSING THE FORMATION OF THE FLUORESCENT EQUIVALENT OF 1.0 nmole 3-OH-BP PER MINUTE AT 37°C

[b]THE INDUCIBILITY (A RELATIVE INCREASE OF AHH OF TREATED TISSUE COMPARED TO CONTROL TISSUE) IS GIVEN PARENTHETICALLY

Table 2. Effects of intratracheal instillation of 200 µg MCA in 0.2% gelatin on pulmonary and hepatic AHH[a] in various strains of mice

STRAIN	LUNG AHH CONTROL	LUNG AHH MCA	LUNG AHH IND.[b]	LIVER AHH CONTROL	LIVER AHH MCA	LIVER AHH IND.
Balb/cMai	0.71	3.1	4.4	19.2	22.2	1.2
C3H/fMai	0.33	2.5	7.7	7.9	7.3	0.93
C57L/J	0.64	3.4	5.3	13.3	27.4	2.1
C57BL/6J	0.28	2.4	8.0	16.1	32.6	2.0
AKR/J	0.28	0.45	1.6	17.5	15.6	0.89
SJL/J	0.19	0.29	1.5	10.8	11.7	1.1
DBA/2J	0.26	0.36	1.4	8.9	8.5	0.95
RF/J	0.41	0.54	1.3	13.5	13.8	1.0

[a]AHH ACTIVITY GIVEN IN TERMS OF UNITS PER g WET WEIGHT TISSUES. A UNIT IS THAT AMOUNT OF ENZYME CAUSING THE FORMATION OF THE FLUORESCENT EQUIVALENT OF 1.0 nmole OF 3-OH-BP PER MINUTE AT 37°C

[b]IND. = INDUCIBILITY; RELATIVE INCREASE OF AHH OF TREATED TISSUE COMPARED TO CONTROL TISSUE

tested, 24 were inducible (51%), and among 42-F2 animals tested, 29 were inducible (69%). These numbers were not statistically different from the 50% and 75% ratios that would be expected if a single autosomal dominant gene were regulating this inducibility.

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Table 3. Genetic regulation of pulmonary AHH in crosses involving the C57BL/6Cum and DBA/2Cum strains of mice[a]

STRAIN	NUMBER OF MICE TREATED	NUMBER OF MICE INDUCIBLE	%
B6	50	50	100
D2	50	0	0
B6D2F1	50	50	100
B6D2F1 x D2	47	24	51
B6D2F2	42	29	69

[a]MICE WERE TREATED WITH 200 µg MCA/.02 ml 0.2% GELATIN SOLUTION INTRATRACHEALLY, AND 24 HOURS LATER, THE PULMONARY AHH WAS ASSAYED. A MOUSE WAS CONSIDERED INDUCIBLE IF, AFTER MCA TREATMENT, PULMONARY AHH WAS 2.5 (+ 0.3) UNITS/g TISSUE AND CONSIDERED NONINDUCIBLE IF PULMONARY AHH WAS 0.30 (+ 0.05) UNITS/g TISSUE. THE SEX OF THE PROGENY PLAYED NO ROLE IN THIS SEGREGATION PATTERN

III. Effect of Tobacco Related Products on Pulmonary AHH Levels

1. Effect of Cigarette Smoke. The pulmonary AHH response of B6 mice exposed to the smoke of one-1A1 cigarette (10% smoke) is shown in Table 4. The lung tissue responded rapidly and selectively to the whole smoke. Peak activity occurred approximately 6 hours posttreatment and remained induced for 24 hours. The intervention of a Cambridge-type filter completely abrogated this induction profile. The use of 2 or 3 cigarettes smoked simultaneously (20% or 30% smoke) gave induction results similar to the use of 1 cigarette (data not shown). Exposure to 1 cigarette-at-a-time for a total of 7 cigarettes (with a 10 min rest between cigarettes) induced pulmonary AHH activity in B6 and C3H/fMai mice, but had only a small effect on D2 mice (Table 5). This smoking schedule resulted in maximal exposure with minimal death if nonpretreated animals were used. Using this schedule, the maximal induction was similar to that induced by 1 cigarette (Table 4): about 2.5 fold in 6 hours. Similar results were also noted using the 1R1 cigarette, i.e., maximal induction occurred within 6 hours after exposure and induction was about 2.5 times that of the sham control or gas-phase treated animals. Similar results were also noted if the holding time was increased from 15 to 28 sec. The 28 sec holding time should allow for maximum deposition of particulate material onto lung tissue (STOCKLEY, Oak Ridge National Laboratory, personal communication, 1974).

Mice could be adapted to higher smoke exposures by pretreatment with only 1 or 2 cigarettes per day for 1 week. By slowly increasing the number of cigarettes (given 1-at-a-time) per day, at 1 month, both D2 and C3H/fMai mice would accept 16 cigarettes per day, 8 consecutive cigarettes in the morning and 8 consecutive in the afternoon. Although slightly more toxic initially, mice would still accept 16-1R1 cigarettes per day using this same schedule. The AHH responses of various tissues of these 2 strains after exposure to 8 cigarettes per day (4 in the morning and 4 in the afternoon) for at least 60 days are demonstrated in Table 6. The chronic high level of smoke seemed to induce only pulmonary tissue; liver, kidney, and small intestinal tissue was unaffected. The data with the intestines was difficult to assess because of the wide mouse-to-mouse variations observed (>200%). Pulmonary tissue from C3H/fMai mice was induced for the whole 18 hours observation period, while the induction of D2 lung tissue seemed to

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Table 4. Effect of exposure to one 1A1 cigarette (10% smoke) on pulmonary AHH levels of C57BL/6Cum mice[a]

	AHH ACTIVITY (UNITS[b]/g TISSUE)		
HR. AFTER SMOKE	WITH FILTER	WITHOUT FILTER	INDUCIBILITY
1.5	0.25	0.24	1.0
3.5	0.21	0.43	1.8
6.5	0.31	0.86	3.6
9.0	0.25	0.53	2.2
12.0	0.25	0.58	2.4
27.0	0.27	0.55	2.3
50.0	0.27	0.38	1.6
74.0	0.27	0.38	1.6
CONTROL	0.24	0.24	

[a]MICE WERE EXPOSED IN A WALTON-TYPE HORIZONTAL SMOKING MACHINE ACCORDING TO THE FOLLOWING 1 MIN CYCLE: 2 SEC PUFF, 15 SEC HOLDING TIME AND 45 SEC PURGE

[b]UNIT IS THAT AMOUNT OF ENZYME CAUSING THE FLUORESCENT EQUIVALENT OF 1 nmole 3-OH-BP/min AT 37°C

Table 5. Effect of 7 consecutive cigarettes[a] on pulmonary AHH activity in various strains of mice

		HOURS[b] POST TREATMENT			
		6		24	
STRAIN	CONTROL	SMOKED	INDUCIBILITY	SMOKED	INDUCIBILITY
C57BL/6Cum	0.20[c]	0.50	2.5	0.45	2.3
DBA/2J	0.25	0.34	1.4	0.32	1.4
C3H/fMai	0.34	0.88	2.6	0.65	1.9

[a]ANIMALS WERE EXPOSED TO CIGARETTE SMOKE USING THE REGIMEN OF ONE CIGARETTE FOLLOWED BY A 10 MIN REST PERIOD FOLLOWED BY ONE CIGARETTE, UNTIL A TOTAL OF SEVEN CIGARETTES WERE SMOKED

[b]HOURS AFTER EXPOSURE TO LAST OF SEVEN (7) 1A1 CIGARETTES

[c]DATA GIVEN IN TERMS OF UNITS AHH ACTIVITY PER g TISSUE. A UNIT IS THAT AMOUNT OF ENZYME CAUSING THE FORMATION OF THE FLUORESCENT EQUIVALENT OF 1 nmole 3-OH-BP PER MIN AT 37°C

have a shorter lifetime and was at background level by 18 hours post-treatment. Responses to the 1R1 cigarettes was similar to that observed for the 1A1 cigarettes.

2. Effect of Cigarette Smoke Condensate Fractions. Fractions of 1A1 cigarette smoke condensate were observed to induce and to inhibit the pulmonary AHH activity of B6 mice 24 hours after IT instillation (Table 7). Fractions B_Ia, B_Ib, N_{MEOH}, and N_{NM} were considered good inducers. The starting material, reconstituted material, B_E, WA_E, WA_I, and N_{CH} were considered weak inducers. Fractions B_W, SA_I, SA_E, and SA_W were actually weak inhibitors of pulmonary AHH activity.

Particular fractions also seemed to have the capability of inhibiting BaP metabolism *in vitro* (Table 8). Data is presented by computing the

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Table 6. Effect of smoking[a] on AHH responses of various tissues of 2 inbred strains of mice

STRAIN	TISSUE	HOURS POST TREATMENT 3 CONTROL	3 SMOKED	3 IND.[b]	6 CONTROL	6 SMOKED	6 IND.[b]	18 CONTROL	18 SMOKED	18 IND.[b]
C3H	LUNG	0.34	1.01	3.1	0.48	0.95	2.0	0.23	0.51	2.2
	LIVER	9.90	10.31	1.1	8.00	9.40	1.2	11.00	13.70	1.2
	KIDNEY	0.06	0.06	1.0	0.07	0.06	0.9	0.06	0.07	1.1
	INTESTINE	1.10	0.63	0.6	0.28	0.30	1.1	0.25	0.35	1.4
D2	LUNG	0.36	0.82	2.3	0.35	1.00	2.9	0.30	0.30	1.0
	LIVER	8.00	10.50	1.3	10.90	9.50	0.9	13.50	14.00	1.0
	KIDNEY	0.08	0.07	0.9	0.06	0.06	1.0	0.07	0.07	1.0
	INTESTINE	0.28	0.99	3.5	0.07	0.08	1.1	0.09	0.07	0.8

[a]ANIMALS WERE PRESMOKED FOR AT LEAST 60 DAYS BY EXPOSURE TO FOUR (4) CONSECUTIVE CIGARETTES GIVEN IN THE MORNING AND FOUR (4) CONSECUTIVE CIGARETTES GIVEN IN THE AFTERNOON. INDICATED TIMES ARE HOURS AFTER EXPOSURE TO LAST OF THE 4 CONSECUTIVE CIGARETTES. CONTROL ANIMALS WERE UNTREATED

[b]IND. = INDUCIBILITY

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Table 7. Effect of fractions of the 1A1 cigarette-smoke-condensate on pulmonary AHH activity of C57BL/6Cum mice[a]

FRACTION NO.[b]	FRACTION	µg	UNITS/g TISSUE[c]	INDUCIBILITY
1	STARTING MATERIAL	2000	.22	1.7
2	RECONSTITUTED	500	.24	1.8
3	$B_I{}^a$	1000	.47	3.6
4	$B_I{}^b$	1000	.32	2.5
5	B_E	50	.19	1.5
6	B_W	500	.07	0.5
7	WA_I	1000	.21	1.6
8	WA_E	500	.24	1.1
9	SA_I	500	.07	0.5
10	SA_E	500	.04	0.3
11	SA_W	2000	.05	0.4
12	N_{MEOH}	2000	.32	2.5
13	N_{CH}	500	.15	1.2
14	N_{NM}	500	.43	3.3
CONTROL	CORN OIL	–	.13	1.0

[a]24 HOURS AFTER IT INSTILLATION OF FRACTION, OR CORN OIL VEHICLE

[b]ARRANGED ACCORDING TO SWAIN et al. (1969)

[c]A UNIT OF AHH ACTIVITY IS THAT AMOUNT OF ENZYME CAUSING THE FLUORESCENT EQUIVALENT OF 1 nmole 3-OH-BP/min AT 37°C

Table 8. *In vitro* effect of cigarette smoke condensate fractions on BaP metabolism in hepatic microsomes[a] from MCA treated B6 mice

FRACTION		[X]/[BaP] TO GIVE 50% INHIBITION[b]
1	STARTING MATERIAL	5.0
2	RECONSTITUTED	5.2
3	$B_I{}^a$	0.8
4	$B_I{}^b$	0.5
5	B_E	3.0
6	B_W	>10.
7	WA_I	5.0
8	WA_E	2.0
9	SA_I	>10.
10	SA_E	>10.
11	SA_W	>10.
12	N_{MEOH}	3.0
13	N_{CH}	ND
14	N_{NM}	1.0
–	7, 8-BENZOFLAVONE	1.0

[a]SOURCE OF MICROSOMES WAS HEPATIC TISSUE FROM MICE PRETREATED 24 HOURS PREVIOUS TO SACRIFICED WITH 80 µg MCA/g BODY WEIGHT GIVEN INTRAPERITONEALLY. SPECIFIC ACTIVITY OF MICROSOMES WAS 0.595 units/mg PROTEIN AND DMSO TREATED CONTROL MICROSOMES WAS 0.583 units/mg PROTEIN

[b]200, 20 and 2 µg OF THE VARIOUS FRACTIONS WERE ADDED TO THE COMPLETE REACTION MIXTURE (EXCEPT BaP), INCUBATED WITH SHAKING AT 37°C FOR 1.0 MIN., AND THEN BaP (20 µg) WAS ADDED AND INCUBATION CONTINUED FOR 20 MIN. DATA GIVEN IN TERMS OF CONCENTRATION OF BaP REQUIRED TO INHIBIT THE FORMATION OF 3-OH-BP BY 50%

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concentration of the material over the concentration of BaP necessary to inhibit enzyme activity by 50% (GOUJON et al., 1973). Using a microsomal preparation from MCA-induced livers, fractions B_Ib, B_Ia, and N_{NM} inhibited BaP metabolism at least as effectively as the known competitive inhibitor of induced AHH, 7,8-benzoflavone. The starting material, reconstituted material, B_E, WA_I, WA_E, N_{MEOH}, and N_{CH} fractions were weak inhibitors, while the B_W, SA_I, SA_E, and SA_W fractions had no effect on BaP metabolism.

E. Discussion

There are major strain-to-strain variations in the AHH levels of inbred strains of mice (THOMAS et al., 1972). Similar variations in AHH activity (or inducibility) also exist in the human system (KELLERMANN et al., 1973a). These variations are under host genetic control in both mice and man, segregating as either a dominant, codominant, or recessive gene in mice, depending on strains employed (ROBINSON et al., 1974), and a single codominant gene in man (KELLERMANN et al., 1973b). Recent information suggests that sensitivity or susceptibility to chemically induced cancers is correlated with the AHH responsiveness of that individual. Individual mice or strains of mice which are AHH inducible are much more sensitive to MCA induced tumors than their noninducible counterparts (KOURI et al., 1973a, 1973b, 1974a); and, in man, individuals with high AHH inducibilities seem much more sensitive to cigarette smoke associated bronchogenic squamous cell carcinomas (KELLERMANN et al., 1973c). In the mouse, hepatic tissue has been used to determine the AHH inducibility of individuals. A valid question would be, does the liver activate (or inactivate) carcinogens for other tissues, or can other organs determine their own sensitivity to chemical carcinogens? Results presented here suggest that at least one other organ, the lung, can be a major determinant in its own ultimate susceptibility to chemically induced cancers.

Using the intratracheal route (to limit the enzymatic response to pulmonary tissue alone) and the B6 and D2 inbred strains of mice whose hepatic AHH responses have been extensively studied, we show in this report: 1. MCA given in a 0.2% gelatin solution induces pulmonary AHH, and this induction is dose dependent; 2. a dose of 200 µg MCA maximally induces pulmonary AHH, but has very limited effect on hepatic AHH levels; 3. pulmonary AHH can be induced in D2 mice by IT administration of MCA, but hepatic AHH levels are never induced; 4. although pulmonary AHH is induced in D2 mice, the levels are very low, with about a 5 fold difference between D2 and B6 pulmonary tissues; and 5. this strain difference is under the same genetic control as that of hepatic tissue, e.g., the highly responsive B6 strain differs from the D2 strain by a single autosomal dominant gene controlling this heightened responsiveness. Results with other inbred strains of mice agree with this contention: strains that are nonresponsive to MCA in their hepatic tissues (THOMAS et al., 1972) are low responders in their lung tissue to IT instilled MCA and vice versa.

These results may seem inconsistent in that the pulmonary tissue of both B6 and D2 mice are induced by IT administration of MCA, agreeing with the results of WIEBEL et al. (1973) and BURKI et al. (1973), yet there seem to be basic differences between these 2 "induction" processes because the responses can be discriminated genetically (Table 3). Recent results from our laboratory (KOURI et al., 1974b) suggest that there is, in fact, a difference between the "induction" of pulmonary tissues of the B6 and D2 strains. Use of the competitive

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inhibitor of induced AHH, 7,8-benzoflavone, and direct quantification of the CO-binding cytochromes from B6 and D2 lung tissue demonstrate that real differences exist between the enzymes in MCA treated B6 and D2 pulmonary tissue. Available data are consistent with the hypothesis that MCA treatment of B6 lung tissue induces the genetically mediated AHH enzyme system associated with the P-448 cytochrome (SLADEK and MANNERING, 1966), and treatment of D2 lung tissue causes the nonspecific proliferation of enzymes that are very similar to the constitutive, or P-450 mediated (GILLETTE et al., 1972), enzymes. Thus, pulmonary AHH is similar, yet different, from hepatic AHH. Similar, in that AHH inducibility seems to be genetically regulated by the same locus, yet different, because there seems to be an organ specific response to MCA in even "noninducible" animals. This response may represent an adaptive response to the environment, since the lungs are constantly exposed to air and dust particles containing polycyclic aromatic hydrocarbons, insecticides, and other aromatic chemicals. This interpretation is in accord with the fact that skin is also slightly inducible in noninducible animals (WIEBEL et al., 1973).

These same strains of mice also respond by increased levels of pulmonary AHH to either whole cigarette smoke (Tables 4, 5, and 6) or particular fractions of condensates derived from this smoke (Table 7). Data are consistent with the observations of WELCH et al. (1971) and MARCOTTE and WITSCHI (1972), who showed that pulmonary AHH can be induced in rats exposed to regular or marijuana cigarettes. The lung seems to possess definite saturation levels for induction via cigarette smoke, for, regardless of schedule, only a 2 to 3 fold induction is observed. Exposure to 1, 2, or 3 cigarettes consecutively, or preexposure for up to 60 days with 8 cigarettes per day, yields quantitatively similar results. The AHH inducible C3H/fMai and B6 strains seem more responsive than the nonresponsive D2 strain (Table 5). However, after chronic exposure, both the C3H/fMai and D2 pulmonary tissues were induced (Table 6). Chronic exposure does not induce AHH levels in the liver, kidney, or intestines. Whether the pulmonary response of chronically smoked D2 mice represents true "induction" (e.g., utilizing the P-448 cytochrome) is presently being evaluated.

Results with the cigarette smoke condensate fractions (Table 7) demonstrate that the components of cigarette smoke that induce (or inhibit) pulmonary AHH can be discriminated. The relatively low inducing potential of whole cigarette smoke may reflect the presence of these inducing and inhibiting components. The BaP containing fraction, N_{NM}, is observed to be an effective inducer of pulmonary AHH and also an effective inhibitor of BaP metabolism *in vitro*. The chemical content of the potent $B_I a$ and $B_I b$ fractions is currently being determined. The phenol- or nicotine-containing fractions (WA_E and B_E) are observed to have little effect on AHH. The severe toxicity observed with the B_E fraction, however, may conceal any interaction between nicotine and AHH. Data in Table 8 nicely corroborate these IT results. Using a partially purified microsomal preparation of hepatic AHH, it was observed that certain fractions (e.g., $B_I a$, $B_I b$, and N_{NM}) are at least as inhibitory of BaP metabolism as 7,8-benzoflavone. Thus, there seems to be a correlation between ability to induce pulmonary AHH and ability to inhibit BaP metabolism *in vitro*. The most likely explanation is that these fractions contain compounds structurally similar to BaP; thus, they are capable of both inducing AHH and inhibiting BaP metabolism (competitively?) *in vitro*. The use of IT instillation of chemicals concomitant with tests for inhibition of BaP metabolism *in vitro* seems to produce rapid and reproducible tests for the detection of compounds that can potentially serve as inducers or substrates for the AHH system. Moreover, preliminary results from

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the laboratory of AMES (U. of California, Berkeley) using these same smoke condensate fractions suggest that certain of these fractions (especially, $B_I a$, $B_I b$, WA_I, and neutral fractions) contain potent mutagenic activity (KIER et al., submitted, 1974). The relative carcinogenecity, and cocarcinogenecity, of these fractions are presently being tested both *in vivo* and *in vitro*.

Published results and preliminary results from our laboratory indicate pulmonary AHH may play a major role in lung cancer susceptibility. NETTESHEIM and HAMMONS (1971) reported conditions for induction of squamous cell carcinoma in inbred strains of mice. These authors utilized the (C57BL X C3H/f) F_1 and the DBA/2 strains of mice and 500 µg MCA (in 0.2% gelatin) given at weekly intervals for 4 to 6 weeks. The AHH inducible F_1 strain (KOURI, unpublished observation) was observed to be much more sensitive to MCA induced squamous cell carcinomas than the AHH "noninducible" DBA/2 strain. Very preliminary results from our laboratory involving IT administration of MCA into parent, F_1, backcross, and F2 animals (involving the B6 and D2 strains) indicate that AHH inducible mice seem to be more susceptible to MCA induced squamous cell carcinoma. Both results are compatible with the idea that the increased susceptibility to chemically induced carcinomas of AHH inducible animals reflects this heightened ability to metabolize chemical carcinogens.

F. Summary

The effects of exogenous factors on lung tissue of inbred strains of mice seem to be largely determined by the enzymatic activity of lung tissue itself. The major enzymatic activity studied in this paper was the inducible enzyme complex, AHH. It was shown that conditions could be developed so that pulmonary AHH levels were singularly effected. IT instillation of MCA (≤200 µg), exposure to whole cigarette smoke, and IT instillation of fractions of cigarette smoke condensate were shown preferentially to induce pulmonary AHH activity, and, in the case of MCA, this response was under host genetic control, segregating as a single autosomal dominant gene in crosses involving the B6 and D2 strains of mice. The small increase in D2 lung tissue following MCA treatment was attributed to nonspecific proliferation of enzymes similar to constitutive AHH, rather than a specific increase of the new P-448-mediated enzymes.

Exposure to whole cigarette smoke from either the 1A1 or 1R1 cigarette, using various exposure schedules, resulted in quantitatively similar increases in pulmonary AHH activity. Pretreatment for 60 days with 8 cigarettes per day did not increase this AHH response. Interposition of a Cambridge filter abrogated this enzyme response. Particular fractions derived from the smoke condensate of the 1A1 cigarette were observed preferentially to induce pulmonary AHH in B6 mice. These same fractions (e.g., $B_I a$, $B_I b$, N_{NM}) also were shown to inhibit BaP metabolism *in vitro*.

Results were discussed in view of the possibility that these enzymatic responses play a major role in the susceptibility of lung tissue to chemically induced cancers.

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The Role of the Host in the Development of *in vivo* Models for Carcinogenesis Studies*

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ABSTRACT

The development of cancer depends upon the integrated response of the host to the carcinogen and to the initial transformation event. Genetic factors determine the host's potential to respond to chemical and viral carcinogens, while endogenous and exogenous environmental factors influence the realization of the genetic potential. In chemical carcinogenesis, inducibility (the capacity to metabolize polycyclic aromatic hydrocarbons (PAH) to the ultimate carcinogen) has been demonstrated to be under genetic control; however, tumors will occur to a lesser degree and after a longer delay when large doses of PAH are administered to noninducible mice. Aryl hydrocarbon hydroxylase induction by non-carcinogenic materials may also influence the effects of PAH carcinogens.

In viral carcinogenesis, evidence points to genetic transmission of the RNA oncogenic viruses with expression of the viral genome under host control. Host control over RNA tumor viruses may be demonstrated by the existence of epigenetic, xenotropic, and pantropic viruses; permissive, restrictive, producer, and nonproducer cell lines; and the presence of viral group specific antigen, infectious virus, and/or neoplasia in the host.

The development of cancer from the initial transformed cells (chemical and/or viral induced) is dependent on the host response which is influenced by numerous factors. Immunosurveillance is probably the first line of defense against these transformed cells. Genetic control of immunocompetence is evidenced by the variety of responses to various antigenetic stimuli in genotypically different strains of mice. Immunosuppressive effects of carcinogens, drugs, infections, etc., appear to make possible the initial act of establishing clones of transformed cells by overriding the immunocompetence of the host. Other factors related to diet, aging, stress, etc., effect the host control over the carcinogenic event and may be related to the increased susceptibility to carcinogens and/or the increase in the incidence of "spontaneous" tumors.

In our laboratory, we have undertaken studies to provide the best possible mouse model system for studying respiratory carcinogenesis. These studies with inbred mice have included the determination of relative susceptibility to various carcinogens, AHH inducibility, type C

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RNA viral expression, immunocompetence when challenged by various antigens, and the immunosuppressive effects of various chemical carcinogens. We plan to further evaluate our model systems with regard to environmental stress factors, cocarcinogenesis, and viral infections. In this way, we hope to establish the integrated host response to the events occurring in respiratory carcinogenesis.

Introduction

The ultimate solution of the cancer problem is dependent not only on the determination of the causes of cancer but also on the mechanisms whereby the host regulates the prevention and surveillance of the carcinogenic event(s) and the ultimate growth of the cancer. We must now ask what are the differences between susceptible and resistant hosts to the causes of cancer. Cancer, as a disease entity, differs in many respects from infectious diseases, in that there is a greater host-parasite involvement. Evidence points to cancer being an epigenetic disease (i.e., its development is dependent on an innate genetic resistance predisposed to gradual failure of control surveillance mechanisms). However, this is not the entire picture, since the response of the host to carcinogenic and subsequent events is an integrated response dependent on the interactions of both genetically controlled mechanisms and internal as well as external environmental factors.

A prerequisite to the etiological studies of any disease is the development of an appropriate animal model system. With the resolution of this problem, rapid progress in treatment and prevention can generally be made. Perhaps the greatest problem in carcinogenesis research has been the lack of a classical experimental animal model system. MEKLER (1973) has suggested that certain fundamental laws of biology pertinent to carcinogenesis have either escaped the attention of researchers or have not yet been discovered. The great strides which have been made in the study of host factors believed relevant to cancer have led us to the conclusion that we must better understand the role of the host in carcinogenesis before we can judiciously select the best animal model.

What species of laboratory animals provides us with the most information regarding those host surveillance mechanisms that may play a role in carcinogenesis? At this time, one would have to agree that there is more available information for various inbred strains of mice. For this reason, we suggest the mouse is still our best hope for selecting one or more model systems where inbreeding can provide the characterization of genetic factors related to carcinogenic susceptibility. There is probably no greater justification for using rats, rabbits, or for that matter, primates to demonstrate the carcinogenic potential of most chemicals. There are other justifications for the use of mice. Much of our knowledge regarding oncogenic viruses comes from mouse studies and has only recently been expanded to other species. In many ways, because of the broad knowledge of mouse genetics, we can come closer in mice to mimicking the situation that exists in man than in any other animal. Mice are easy to handle and require less space than most other animals; therefore, statistically significant results can be obtained by the use of large populations. Their relatively short life span of 2 to 3 years also makes them ideal subjects for lifetime studies. Their extraneous virus flora have been relatively well characterized and can be controlled by quarantine procedure. Their "spontaneous" tumor incidences have also been characterized.

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There are of course various disadvantages, as is true with any model system. For respiratory carcinogenesis studies, mice have the disadvantage of being obligate nose breathers and this may present a different picture from that seen in man. Rats and mice have 4 nasal glands (vs 1 in man) which discharge fluids upon breathing noxious chemical or physical irritants. Mice also have more goblet cells per surface area of the respiratory epithelium (WYNDER and HOFFMANN, 1969). The organs and blood supply in mice are also small, thus presenting the disadvantage that only a limited number of studies can be undertaken with any one animal.

For the past 10 years, our laboratory has been actively defining certain parameters of viral-chemical carcinogenesis. Fortunately, other laboratories have pursued other aspects of genetically controlled surveillance mechanisms, which play important roles in cancer. It is our purpose in this paper to look at the integrated host (i.e., the inbred mouse) in an attempt to pull together many of the factors we feel play a role in the selection of the best possible animal model system for respiratory carcinogenesis. Our discussion of the mouse model system presents several aspects of host control over susceptibility:

- Susceptibility to chemical carcinogens
- Chemical carcinogen metabolism
- Viral etiology of cancer
- Tumor immunology
- Other genetic controls of neoplastic development
- Cells at risk to carcinogens and DNA repair

Chemical Carcinogenesis Susceptibility

During the past 7 years we have concentrated primarily on the characterization of various genotypically different mouse strains as to their susceptibility to 3-methylcholanthrene (MCA), 7,12-dimethylbenz(a)anthracene (DMBA), and benzo(a)pyrene (BaP) when given subcutaneously to female mice 4 weeks of age (WHITMIRE et al., 1971; WHITMIRE and SALERNO, 1972; KOURI et al., 1973b). These studies were based on an 8 month observation period, since most tumors occur during the first 5 to 6 months. The doses selected were relatively small, since our primary aim was to determine relative susceptibility in a reasonable period of time with as few adverse effects on the host as possible. The basis for comparison has been the cumulative tumor incidence, latency period (SHIMKIN and ANDERVONT, 1940), carcinogenic index (CI), (% cumulative tumors divided by the average days latency X 100)(IBALL, 1939), and tumor inducing dose in 50% of the animals in the defined observation period (TuD_{50}), (REED and MUENCH, 1938). We studied those mouse strains (inbred and random bred) most frequently used in cancer research. We have found correlation of the extensive literature difficult due to the numerous variables that inevitably exist between laboratories. Our results from studies carried out over an extended period using the same techniques, carcinogen dose, vehicle, and mice of comparable age and sex are presented in Table 1. Table 2 gives some of the host genetic factors for these inbred strains as a ready reference for later discussions.

These earlier studies with MCA have demonstrated a wide variation in the tumor incidence as well as average latency period among the genotypically different strains. Although the most susceptible strains generally develop tumors in the least amount of time, this is not

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necessarily the case. For this reason the carcinogenic index (CI) has been utilized as a valuable index in rating the relative susceptibility of the various mouse strains (IBALL, 1939; WHITMIRE and SALERNO, 1972a). Probably the most outstanding examples of variation between tumor incidence and latency are the C3H/fMai and the C3H/HeJ, whose average latency is 14 weeks in each incidence but which produce 93% and 62% tumors respectively. Another example is that of the C3H/HeJ mice (14 weeks latency), which produce only 62% tumors, and the C57BR/cdJ strain, which produce 67% tumors with a relatively long average latency (22 weeks). These studies demonstrate that, although NIH Swiss, Ha/ICR, CFW, and CF-1 random bred strains have been used extensively in carcinogenesis studies, they are not as susceptible as a number of inbred strains. To define the relative susceptibility to MCA carcinogenesis still further, several dose levels were used and we found the C3H/fMai mouse strain to be consistently more susceptible than the other strains tested (Table 3). These studies also demonstrate a difference in mice of the same strain obtained from different sources. This is one variable in carcinogenesis studies of which most investigators are not aware and undoubtedly accounts for some of the variations in reported findings from one laboratory to another. The C57BL/6 mice from 3 sources act as entirely different strains (WHITMIRE and SALERNO, 1972a).

Table 1. Subcutaneous chemical carcinogenous characterization of various mouse strains with 150µg MCA

MOUSE STRAIN	TUMOR INCIDENCE Tu/T	%	AV. TU. LATENCY (WKS)	C.I.[a]	AHH[b] INDUCIBILITY	gs-1 ANTIGEN INCIDENCE P/T[c]	%	INFECTIOUS TYPE-C VIRUS
INBRED								
C3H/f Mai	51/55	93	13	102	9	16/23	70	±
C57BL/6 Cum	18/20	90	17	76	10	3/26	12	–
C58/J	20/23	87	16	76	10	10/10	100	+++
C57BL/10ScSnJ	22/27	81	18	64	12	0/14	0	–
BALB/cCr (Mai)	35/49	71	19	53	8	5/23	22	±
C57BR/cdJ	18/27	67	22	44	7	2/18	11	–
C3H/HeN	33/53	62	14	63	10	13/24	50	+
SWR/J	11/22	50	23	33	1	6/10	60	–
129/J	14/30	47	27	25	1	0/15	0	–
C57L/J	13/28	46	20	33	6	0/13	0	–
AKR/J	20/51	39	26	21	1	9/9	100	+++
SJL/J	13/35	37	22	24	1	11/15	73	++
DBA/2J	5/35	14	28	7	1	12/12	100	+
DBA/1J	6/53	11	27	6	1	4/8	50	+
RANDOM BRED								
SWISS-WEBSTER (N)	26/35	74	17	62	1	13/25	52	–
Ha/ICR (RPMI)	46/63	73	19	55	1	14/23	61	++
CFW	21/29	72	17	61	1	9/13	69	+
CF-1	37/66	56	23	33	1	15/15	100	++
SNELL/Mai	9/25	36	24	14	1	11/15	73	+

[a] $\text{CARCINOGENIC INDEX} = \frac{\%\ \text{TUMORS}}{\text{AV. DAYS LATENCY}} \times 100$

[b] $\text{AHH INDUCIBILITY} = \frac{\text{AHH LEVELS FROM MCA-TREATED MICE}}{\text{AHH LEVELS FROM TRIOCTANOIN TREATED MICE}}$

HEPATIC AHH LEVELS WERE DETERMINED 24 HOURS AFTER IP ADMINISTRATION OF 80 µg MCA/g BODY WEIGHT OR 0.05 ml TRIOCTANOIN. CONSTITUTIVE AHH LEVELS WERE SIMILAR FOR EVERY STRAIN TESTED

[c] $\frac{P}{T} = \frac{\text{NUMBER OF POSITIVE SAMPLES BY CF TEST}}{\text{TOTAL SAMPLES TESTED}}$

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Table 2. Genes influencing AHH inducibility, tumor virus histocompatibility, and immunological responses

MOUSE STRAIN	GENE DESIGNATION[a]						IMMUNOLOGICAL RESPONSES[b] H-2 ASSOCIATED						NON H-2 ASSOCIATED			
							Ir-1 GENES SYNTHETIC POLYPEPTIDE COPOLYMERS (,)-A--L				Ir-Ig GENES BALB/c MYELOMA PROTEIN		Ir-3 GENES POLYPEPTIDES (,)-Prol--L		SC[c]	
	Ah	Fv-1	Fv-2	H-2	Ir-1	Ir-3	(T,G)	(H,G)	(Phe,G)	GAT_{10}	IgG	IgA	(H,G)	(T,G)	A	C
C3H/f	b	n	s	k	a	H9	L	H	H	H	L	H	H	H		
C57BL/6	b	b	r	b	b	H9	H	L	H	H	H	L	H	M		
C58	b	n	r	k	a	H9	L	H	H	H	L	H				
C57BL/10ScSn	b	b	r	b	b	H9	H	L	H	H	H	L				
BALB/c	b	b	s	d	a	H9	Mv	Mv	H	H	L	L				
C57BR/cd	b	n	r	k	a	H9	L	H	H	H	L	H				
C3H/He	b	n	s	k	a	H9	L	H	H	H	L	H	H	L		
SWR	d	n	s	q	c	H9	L	L	H		L	L	H	H	H	H
129	d	n	s	b	a	H9	H	L	H	H	H	L				
C57L	b	n	r	b	a	H9	H	L	H	H	H	L				
AKR	d	n	s	k	d	H-	L	H	H	H	L	H	H	L		
SJL	d	n	s	s	b	H9	L	L	L	neg	H	H	H	H	H	M
DBA/2	d	n	s	d	c	H9	Mv	Mv	H	H	L	L	H	L	H	M
DBA/1	d	n	s	q	c	H9	L	L	H		L	L	L	L	H	L

a STATTS, 1972

b McDEVITT AND LANDY, 1972

L = LOW
M = MEDIUM
Mv = MEDIUM VARIABLE
H = HIGH

[c]STREPTOCOCCAL CARBOHYDRATE ANTIGENS

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Table 3. Comparison of tumor incidence, mean latency, CI and TuD_{50} in various strains of mice treated subcutaneously with various doses of MCA at 4 weeks of age

MOUSE STRAIN	9.38 µg TUMOR INCIDENCE Tu/T	%	MEAN CI LAT (WKS)		37.5 µg TUMOR INCIDENCE Tu/T	%	MEAN CI LAT (WKS)		150.0 µg TUMOR INCIDENCE Tu/T	%	MEAN CI LAT (WKS)		TuD_{50} µg MCA
AHH INDUCIBLE													
C3H/fMai	9/28	32	23	20	24/29	83	18	68	27/30	90	13	96	21
C3H/AnfCum	9/29	31	21	21	16/29	55	17	46	25/29	86	14	89	57
C57BL/6Cum	10/28	36	21	21	14/27	52	23	32	26/27	96	17	83	26
C57BL/6Mai	–	–	–	–	12/30	40	21	28	20/27	74	19	50	61
C57BL/6J	–	–	–	–	14/29	48	20	34	16/27	59	20	44	64
BALB/cCR (Mai)	3/30	10	27	7	17/28	61	20	45	23/28	82	16	71	34
BALB/cSPF (Mai)	–	–	–	–	20/30	67	17	57	26/31	84	12	98	≥38
C57BL/10ScSn	–	–	–	–	12/30	40	23	25	22/27	81	18	64	41
AHH NON-INDUCIBLE													
	150.0 µg				300.0 µg				500.0 µg				
129/J	14/30	47	27	25	16/28	57	21	39	20/28	71	22	46	203
DBA/2J	10/24	42	25	24	14/24	58	22	38	21/24	88	21	59	212
DBA/1J	4/27	15	25	9	13/24	62	25	35	10/19	53	23	33	238

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The susceptibility of an animal to one carcinogen does not insure its responsiveness to another carcinogen. Therefore, studies were undertaken with DMBA and BaP in selected strains. As seen in Table 4, the C3H/fMai strain is highly susceptible to all 3 carcinogens, while the other strains are relatively insensitive to tumor induction with BaP and show varying sensitivity to DMBA. In all instances, the latency periods were longer with DMBA and BaP than with MCA. Although all polycyclic aromatic hydrocarbons are believed to be metabolized by the AHH enzyme system, there is evidence that their metabolism pathways are slightly different (NEBERT et al., 1973), which may account for genetic differences in the strains tested.

Chemical Carcinogen Metabolism

In order for polycyclic aromatic hydrocarbons (PAH) to exert cell transformation, mutagenicity, and tumor induction, they must be metabolized to water soluble active forms (e.g., the epoxide), (MARQUARDT and HEIDELBERGER, 1972; HUBERMAN et al., 1972) by the microsomal bound, mixed function oxidases of which aryl hydrocarbon hydroxylase (AHH) enzymes are a major system found in the tissues of man (KELLERMANN et al., 1973) and animals (GELBOIN, 1967). Constitutive enzyme levels are normally detectable without induction; however, the inducibility of AHH is associated with the carcinogenic effects of PAH (SELKIRK et al., 1971). The AHH system is inducible by a variety of endogenous chemicals (corticorsteroid hormones and bilirubin), as well as exogenous chemicals (barbiturates, insecticides, and PAH); therefore, these enzymes obviously may function as a two-edged sword. Our studies with various strains of mice have demonstrated a direct correlation between inducibility of AHH activity and 150µg MCA subcutaneous carcinogenesis (KOURI et al., 1973b). The results are summarized in Table 1. If, however, one gives higher doses of MCA (Table 3) to noninducible mice, the incidence of tumors can be increased, while the latency period remains at 5 to 6 months and the CI index below 60. The TuD_{50} dose is 3 to 11 times that of the inducible mice.

The role of AHH appears highly specific for each PAH. Thus, the metabolism of MCA, DMBA, and BaP does not necessarily follow the same pathways indicated by subcutaneous and skin carcinogenesis studies (KINOSHITA and GELBOIN, 1972; KOURI et al., 1973b; NEBERT et al., 1973). These differences in the carcinogenic effects (Table 4) indicate that the C3H/f mouse was the only strain capable of handling all 3 carcinogens equally well. It would appear that these variations in the AHH system could be clarified by studying congenetic crosses between the C3H/f strain and another strain giving low levels of tumor induction with BaP and DMBA and might help explain important etiological differences in chemical carcinogenesis.

The inducibility of the AHH system by various chemicals has been shown to be under host regulation. Susceptibility to MCA induction segregates as a single autosomal dominant gene in crosses involving the C57BL/6 (B6) and DBA/2 (D2) strains of mice (THOMAS et al., 1972; NEBERT et al., 1972b; GIELEN et al., 1972b). The B6 is the prototype inducible strain and its allele Ah^b designates the dominant gene. The D2 strain is the prototype strain for the recessive Ah^d allele. We utilized this mouse genetic system to extend our observations on the relationship between AHH inducible and sensitivity to MCA tumorigenesis (KOURI et al., 1973a, 1974c). The results in Table 5 demonstrate that inducible animals were approximately 10 times more sensitive to MCA carcino-

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Table 4. Tumor incidence, mean latency, and CI in various strains of mice (females) inoculated subcutaneously with 150µg MCA, DMBA and BaP

	150 µg MCA				150 µg DMBA				150 µg BaP			
MOUSE STRAIN	Tu/T	%	MEAN LAT (WK)	CI	Tu/T	%	MEAN LAT (WK)	CI	Tu/T	%	MEAN LAT (WK)	CI
C3H/fMai	51/55	93	13.2	100	24/25*	96	17.5	78	20/24*	83	18.3	65
B10.BR/J	26/28	93	16.3	83	16/29	55	21.0	37	3/25	12	21.7	8
C57BL/6Cum	18/20	90	16.5	78	5/17	29	22.4	19	2/24	8	19.0	7
C57BL/10ScSn	22/27	81	18.1	64	10/24	42	22.8	26	4/29	14	24.0	8
129/J	14/30	47	27.0	25	11/33	33	26.5	18				
Snell/Mai	9/25	36	23.6	24	8/23	35	24.0	29	3/29	10	22.0	6

*THESE ANIMALS WERE MALES

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genesis than noninducible animals when comparing the CI values. In every case where a tumor was observed on a noninducible animal, it occurred late in the observation period after tumor development had ceased in the inducible animals. It is assumed that some metabolism of this carcinogen took place at a much slower rate (possibly by the constitutive levels of AHH enzymes), leading to cell transformation and tumor induction.

The significance of AHH induction in the transformation of tissue culture cell lines by PAH carcinogens has been demonstrated (KOURI et al., 1974b). Only those cell lines potentially sensitive to chemically induced transformation possessed the particular type of metabolism involving the AHH inducible enzymes. The carcinogenic effect of a hydrocarbon is probably determined by the amounts activated to the carcinogenic form. The low levels of these enzymes in many tissue culture cell lines are probably a factor in the inability to obtain chemically induced transformation.

Enhancement and interference in the metabolism of chemicals by the AHH system can occur. CONNEY (1974) has demonstrated that oral treatment with MCA, DMBA, and BaP enhanced the metabolism of intravenously (IV) administered radioactive BaP in rats, while phenobarbital (which stimulates AHH induction) did not enhance MCA, BaP, or DMBA metabolism. Chronic administration of BaP stimulates the metabolism of radioaction BaP. These observations are of interest since treatment of rodents with AHH inducers provides protection for the carcinogenic effects of BaP, DMBA, N-2-fluorenylacetamide, 4-dimethylaminostibene, urethane, aflatoxin, diethylnitrosamine, and aminoazo dyes (CONNEY, 1974). THAMAVIT et al. (1974) have also reported that treatment with 3 carcinogens at one time decreases their carcinogenesis in rats and was believed due to an interference phenomena. WEBER et

Table 5. Relationship of hepatic inducibility and subcutaneous tumor induction with 150µg MCA in C57BL/6 x DBA/1, F1, and F2 mice

	AHH INDUCIBLE		AHH NON-INDUCIBLE	
	TUMOR INCIDENCE		TUMOR INCIDENCE	
MOUSE STRAINS	Tu/T	%	Tu/T	%
AHH NONSEGREGATING:				
B6 (Ah^b/Ah^b)	23/29	79		
D2 (Ah^d/Ah^d)			2/30	7
F1 (Ah^b/Ah^d)	54/90	60		
F1 x B6 (Ah^b/Ah^d and Ah^b/Ah^b)	81/94	86		
TOTALS:	158/213	74	2/30	7
AHH SEGREGATING:				
F1 x D2 (Ah^b/Ah^d and Ah^d/Ah^d)	15/24	75	5/34	15
F2 (Ah^b/Ah^b Ah^b/Ah^d Ah^d/Ah^d)	23/25	92	2/21	10
TOTALS:	38/45	84	7/55	13
TUMOR TOTALS:	196/258	77	8/85	11
AV. DAYS TUMOR LATENCY:	131		195	
CARCINOGENIC INDEX:-	59		6	

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al. (1974) recently reported that nicotine reduced the metabolism of benzopyrene in tobacco smoke thus demonstrating interference between chemicals metabolized by the same enzyme system.

Not all known carcinogens belong to the group of chemicals known as PAH and their metabolism proceeds along different pathways. They have not been studied as extensively as the PAH group and the relationship to genetic patterns of susceptibility to carcinogenesis has not been determined. The dimethylase associated with the metabolism of the dimethylnitrosamines (DMN) may bear a mirror-image relationship to the AHH system, since high levels of induced AHH may act as potent repressors of DMN-dimethylase activity (VENKATESAN et al., 1971). This suggests that DMN may be a more potent carcinogen in AHH non-inducible animals. We are undertaking studies in AHH inducible and noninducible animals to test this hypothesis.

Virus Etiology of Cancer

Although a number of tumor viruses have been isolated from a variety of animal species since ROUS (1911) first discovered the avian sarcoma virus, the concept of viral etiology of cancer has been untenable for many scientists. The development of inbred mouse strains produced high and low incidence leukemia strains and ultimately led to the demonstration that RNA tumor viruses could be transmitted both horizontally and vertically (GROSS, 1944, 1950, 1970). Transmission can take place by congenital infection of the germ cells or via the placenta or milk; however, the usual mode of spread appears to be by genetic inheritance from one generation of animals or cells to the next as DNA copies of viral RNA integrated into the genetic material of the cells (WEISS, 1973; BUFFET et al., 1969; HILGERS et al., 1972).

Two concepts have been proposed for the origin of RNA tumor viruses: the oncogene hypothesis of HUEBNER and TODARO (1969) (TODARO and HUEBNER, 1972) and the protovirus hypothesis of TEMIN (1971, 1972). The oncogene theory suggests that viral genetic material is present in normal cells expressed as infectious virus or as noninfectious viral subunits (as viral group specific (gs) antigen) and that a noninfectious portion of the viral genome (the oncogene) is responsible for cellular transformation and cancer. The protovirus hypothesis differs from the oncogene hypothesis by suggesting that infectious viral genetic information is transferred by transcription and reverse transcription and, in combination with mutation or recombination events, produces neoplastic transformation.

Regardless of the hypothesis for the origin of the RNA tumor viruses, there are certain cellular controls which govern their expression. These controls will vary somewhat between endogenous and exogenous viruses; therefore, it is pertinent that some of the properties of each be considered. Endogenous viruses are transmitted vertically, either as viral genome or as infectious virus by congenital means. Multiple copies of the virogene are present in the DNA of all somatic and germ cells of all animals in a species. The type of viral expression is under cellular control and may be present as gs antigen, defective virus, or complete virus capable of growth under proper conditions with the production of reverse transcriptase (RT). Clonal lines established from these tissues will either spontaneously release virus or induce virus release after varying intervals of cultivation, depending on the original viral expression and the strength

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or degree of cellular control. Induction can also be accomplished with 5'-iododeoxyuridine (IudR) or 5-bromodeoxyuridine (BudR). Complete virogene is known to be present in chickens, Chinese and Syrian hamsters, mice, rats, cats, pigs, and baboons and has been demonstrated by single cell clones with release of infectious virus. These cells with endogenous virogene are generally resistant to exogenous infection by the homologous endogenous virus. The expression of endogenous virus is influenced by the genetic properties of the virus and the cell as well as exogenous factors, such as radiation, chemical carcinogens, etc. Exogenous viruses differ from endogenous viruses in that they are spread horizontally as infectious virus (with evidence of RT) from animal to animal or cell to cell.

The characterization of the mouse type C viruses has recently been reviewed by SARMA and GAZDAR (1974). The type C RNA viruses from mice can be divided into 2 groups. The sarcoma viruses (MSV) produce solid tumors *in vivo* and transformed or cytopathogenic foci *in vitro*. These have been isolated rarely from laboratory adopted stocks of mouse leukemia viruses (MuLV), (HARVEY, 1964; MOLONEY, 1966; KIRSTEN and MAYER, 1971) or from several spontaneously occurring mouse sarcoma (FINKEL et al., 1966; GAZDAR et al., 1972). MSV can transform cells and release virus or they can fail to release virus, as seen in the nonproducer cell lines where the viral genome is integrated into the genetic material of the host cell. In such cases the viral genome can be rescued by superinfection with MuLV, which provides the envelope for the defective MSV. The mouse leukemia viruses (MuLV) are noncytopathogenic *in vitro* when propagated in permissive cells. *In vivo*, some produce leukemia while others fail to produce evidence of any neoplastic potential under the test conditions. They have been isolated from spontaneous and chemically induced solid tumors.

Recently, it has been shown that 2 classes of murine, RNA, type C viruses exist, based on their ability to replicate in mouse tissue. Those which will not replicate in mouse tissue but require rabbit, human, cat, etc., tissue are called xenotropic viruses (X-tropic), (LEVY, 1973) or S-tropic (SHERR et al., 1974). These viruses have typical murine type C antigenic markers but differ distinctly by nucleic acid hydridization from the N-tropic MuLV (BENVENISTE et al., 1974). The significance of the X-tropic viruses has not been determined at this time although they are apparently widespread. Their presence in the mouse with the apparent inability to propagate at least *in vitro* in mouse tissues presents an interesting type of genetic control that requires additional study. The implications of such viruses in humans provide a possible explanation to our inability to propagate a human cancer virus. MuLV, which replicate preferentially in mouse tissues, have been classified as ecotropic viruses (LEVY, 1974), and make up the group of murine viruses for which considerably more information is available. It is these viruses that will be discussed in this paper.

Vertical transmission of virogene information bypasses the host controls associated with infectious viral (but not necessarily oncogene) expression. Such host controls over infectious virus are common to vertically and horizontally transmitted infectious virus expression. Evidence of viral genetic information in the absence of infectious virus has been demonstrated by several systems. Virus-like molecule sequences were reported by HAREL et al. (1967) in the DNA of uninfected murine cells. CHASE and PIKO (1973) and VERNON et al. (1973) observed C-type particles, and gs antigen (HUEBNER et al., 1970a) has been demonstrated in embryonic tissues of mice. The $Gross_{IX}$ MuLV associated antigen was demonstrated by STOCKERT et al. (1971). Spon-

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taneous and induced appearance of MuLV from clones of nonproducer cell lines has been demonstrated by TODARO (1972), AARONSON et al. (1969, 1971).

Based on our present technology for detecting type C RNA viral expression there appear to be 4 categories of mouse strains: 1. the C57L mouse expresses no viral antigen or infectious virus; 2. the NIH Swiss mouse has only the gs-1 antigen and no G_{IX} antigen, and the 129 strain expresses some gs-1 and G_{IX} antigen (neither of these 2 mice has infectious ecotropic MuLV, although type C particles have been observed in NIH Swiss mice); 3. certain mice have a low incidence of early ecotropic MuLV expression as gs-1 antigen that gradually increases and is accompanied by low levels of infectious MuLV expression as seen in the BALB/c mouse (PETERS et al., 1972a); and 4. a high level of infectious virus is detected early in life, accompanied by a high incidence of leukemia as demonstrated in AKR, C58, and C3H/Fi.

Several genes have been associated with endogenous virus expression; however, their roles have not been fully evaluated and may be related more to the expression of neoplasia than the virus per se. The TL antigen, determined by the Tla locus, may represent a viral genome since it appears only on leukemic cells and thymocytes of certain strains (BOYSE and OLD, 1969). The G_{IX} antigen is found on thymocytes and lymphocytes, but all murine leukemic cells are not G_{IX}^{+} (STOCKERT et al., 1971). TAYLOR et al. (1971) described 2 independent genes (no designation made) for gs antigen expression in the AKR mouse and postulated one locus for gs antigen expression and another for infectious virus in the AKR X C57BL/6 F2 cross. It is possible these 2 genes may be the same as the V_1 and V_2 loci for complete virus production in the AKR mouse described by ROWE (1972). These V-loci and the Ind locus (STEPHENSON and AARONSON, 1972a, 1972b) predispose cells to virus induction by IudR and BudR (ROWE et al., 1971). TAYLOR et al. (1973) also described the Mlv-1 allele in the C57BL/10 and DBA/2 strains as a determinate of gs antigen expression. Another gene (Fv-2) has been characterized for host control over propagation of the spleen focus-forming virus (SFFV) component of Friend virus complex. The susceptibility phenotype ($Fv\text{-}2^{s}$) is dominate and the $Fv\text{-}2^{r}$ denotes absolute resistance. This linkage is unrelated to the H-2 linkage and has no direct influence on the lymphatic leukemia virus of Friend (MCDEVITT and LANDY, 1972).

Interferon has been shown to be a potent antiviral agent and its production has been shown to be under host control (deMAEYER and deMAEYER-GINGNARD, 1969). This control is apparently related to the host response to various interferon inducers (BARON, personal communication, 1974) therefore demonstrating another variation in host control of infectious virus expression.

The best defined of the host cellular control genes for MuLV is that governing the replication of infectious virus. This spreading factor influences the ability of endogenous as well as exogenous ecotropic viruses to express themselves as infectious virus. The Fv-1 locus controls the host range permissiveness of mouse cells for the replication of MuLV (PINCUS et al., 1971a, 1971b). HARTLEY et al. (1970) demonstrated 3 groups of MuLV based on their ability to grow more efficiently in NIH Swiss (N) cells ($Fv\text{-}1^{n}$) or BALB/c (B) cells ($Fv\text{-}1^{b}$) and designated this predilection of the viruses as "N- and B-tropic" and NB-tropic" for those viruses that grow equally well in both types of cells. The tropism of the various mouse strains and their embryonic tissue culture cells have been classified as N-type or B-type. In the case of the MSV, the tropism is dependent on the tropism of the helper

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virus. Mice strains that develop early leukemia belong to the Fv-1^n group (AKR, C58, C3H/Fi). These mice also carry the V_1 and V_2 alleles for high incidence activation of the endogenous MuLV. See Table 6 for summary of host genes associated with RNA type C viral expression control.

These type C viruses and the various host control mechanisms have been shown to play a role in the incidence of "spontaneous" (PETERS et al., 1972b) and virally induced neoplasia (LILLY and PINCUS, 1973; ROWE, 1972). They have been postulated to be switched on by chemical carcinogens (HUEBNER et al., 1970b, 1972; MEIER and MYERS, 1973); however, their significance in chemical carcinogenesis is still open to speculation. If one does undertake induced viral-chemical carcinogenesis studies, one must be cognizant of the susceptibility of the mouse strain not only to the chemical carcinogen used but also to the virus selected for these studies. It is also necessary to recognize the importance of endogenous viruses and the ensuing natural incidence of early or late development of leukemia when selecting inbred strains and congeneic strains for carcinogenesis studies related to the interaction of spontaneous and induced neoplasia (WHITMIRE et al., 1972b, 1972c, 1973a; SALERNO et al., 1973).

To determine the occurrence and concomitants of viral expression during MCA carcinogenesis in the various mouse strains, we have followed the incidence of gs antigen and infectious virus in the induced tumors (WHITMIRE et al., 1971, 1973b; WHITMIRE and SALERNO, 1972a). These results are summarized in Table 1. There is no significant correlation between susceptibility to MCA and the presence of viral expression as gs antigen or infectious virus. Other studies have confirmed this finding with DMBA and BaP (KOURI et al., 1973b). The gs antigen expression in chemically induced tumors follows the same pattern as that of the spleens of normal animals (MYERS et al., 1970) and could be related to the degree of expression that increases with age, as observed in the BALB/c mouse (WHITMIRE et al., 1973b).

Tumor Immunology

The significance of the host's ability to respond immunologically to the events of carcinogenesis was recognized early, yet today not all the ramifications of tumor immunology are understood. Further advances in technology and more knowledge regarding the integrated nature of host defense mechanisms might shed some light on this complex area. There exists the dichotomy between the healthy immune stimulus that provides for elimination of the initial transformed cells or the holding action in the host-parasite relationship, and the unhealthy condition where the immunological response enhances tumor growth. A point in cancer research has been reached when we must use the available knowledge regarding immunological competence for the selection of our animal models. It is my purpose to review some of these factors to be defined or at least recognized as existing in the animal models selected for the study of viral, chemical, and viral-chemical carcinogenesis. Sweeping conclusions regarding carcinogenic events can no longer be made in model systems without considering the integrated host reactions.

The metabolism of chemical carcinogens and factors related to viral etiology have already been considered; however, it will be necessary to consider the immunological response involved in those 2 facets that

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Table 6. Genes influencing ecotropic mouse tumor virus expression (TOOZE, 1973)

ALLELE	PHENOTYPE	EXPRESSION	EXAMPLE STRAIN	REFERENCE
Tla	TL ANTIGEN	+	A, C58	BOYSE & OLD (1969)
		−	AKR, C57BL/6	
NOT DESIGNATED	gs ANTIGEN	+	AKR	TAYLOR, MEIER, MYERS (1971)
		−	C57L	
NOT DESIGNATED	INFECTIOUS MuLV	+	AKR	TAYLOR, MEIER, MYERS (1971)
		−	C57L	
V_1	N-TROPIC MuLV INDUCTION	V_1	AKR, C58, C3H/Fi	ROWE (1972)
		v_1		
V_2	N-TROPIC MuLV INDUCTION	V_2	AKR, C58, C3H/Fi	ROWE & HARTLEY (1972)
		v_2		
Ind	N-TROPIC MuLV INDUCTION	+	BALB/c	STEPHENSON & AARONSON (1972b)
		−	NIH-SWISS	
Fv-1	TISSUE TROPISM FOR VIRAL REPLICATION	n	AKR, C58	ROWE & HARTLEY (1972)
		b	C57BL/6, BALB/c	STEPHENSON & AARONSON (1972a)
		nb	NZB	
H-2	EARLY LEUKEMIA	k	AKR	BOYSE, OLD, STOCKERT (1972)
	LATE LEUKEMIA	b	AKR/H-2^b	
G_{1X}	G_{1X} ANTIGEN	+	129	BOYSE, OLD, STOCKERT (1972)
		−	C57L	
Mlv-1	gs ANTIGEN	a	C57BL/10, DBA/2	TAYLOR, MEIER, HUEBNER (1973)
		b		

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allow the initial carcinogenic event to establish itself. Immunosuppressive effects of virus (SHEARER et al., 1973) and chemical carcinogens (STJERNSWÄRD, 1965; REES and SYMES, 1973; MATSUOKA et al., 1972; PARMIANI et al., 1971; BALL et al., 1966; BALL, 1970) undoubtedly play a significant part in the initial carcinogenic event. We are currently addressing ourselves to defining the immunosuppressive effects of various chemical carcinogens given intratracheally for the induction of lung cancer in several strains of mice (DEMOISE et al., 1974). Just as NETTESHEIM and HAMMONS (1971) have shown differences in susceptibility to lung carcinogenesis by MCA, STUTMAN (1969) indicates differences in the immunosuppressive effects in genotypically different strains. Based on our studies with MCA we would anticipate that both differences in tumor induction and immunosuppression may be correlated with the AHH inducibility of the various mouse strains (KOURI et al., 1973b).

The possible mechanisms whereby chemical carcinogens bring about carcinogenic events may be dependent not only on the transforming events but also on immunodepression, that allows these transformed cells to bypass the immunological defense mechanisms of the host. Immunosuppression by chemical carcinogens may, in fact, not be a single event, but cumulative, recurring events that allow frequent bypassing of host defense mechanisms and consequently slow interrupted but progressive growth of tumor cells.

The term "immunologic surveillance" coined by THOMAS (1959) and further expounded by BURNET (1970a, 1970b) denotes the idea of "seeking out and destroying" transformed cells by immunological means. This idea may not be totally correct, because rather than destruction, a "holding action" (LAPPE, 1971, 1972) may develop until such time as events in the integrated host's defense provide a favorable climate for the growth of the transformed cells (HESTON, 1963). This "sneaking through" event of some transformed cells that ultimately leads to the development of cancer is more of apparent scientific basis than it initially appeared. "Sneak through" has been thought to occur due to the location of the transformed cells in sites not exposed, or exposed less frequently to concomitant immunity. This unequal exposure to immune mechanisms is also believed to play a role in the site of metastites (VAAGE et al., 1971).

Another factor allowing "sneak through" of initial transformed cells is the low antigenic profile of some tumor cells as well as the low antigen load presented by only a few cells. For tumor cells to escape immune surveillance there must first be a barrier to escape. Non-immunogenic or low immunogenic tumors may not be capable of surveillance by immunological means. One must then define what we mean by "nonimmunogenic". Is it that these tumor cells are so like normal cells that they cannot be recognized as foreign by the host? Self-non-self discrimination is not fully understood and is intertwined with immunological tolerance, immunological paralysis, and immunological recognition. Is it that the host is tolerant to these cells since they contain certain embryonic antigens or endogenous viral genome antigens? Or, is it that the host is not capable of recognizing these antigens due to genetic variations within the species? This is where the inbred laboratory animals help us understand the role of genetic determinants in the immune process and, ultimately, to understand the events of neoplastic initiation and developments. Genetic variation in the immunological response can definitely influence neoplasia. Many of the differences in specific immunological responses have been linked to the histocompatibility gene in the mouse, rat, and guinea pig. Viral susceptibility has also been closely associated with the H-2 locus and such variations in susceptibility may be shown to be related, at least

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in part, to inability to produce an immunological response in susceptible mice. These immune responses are under the control of individual dominant autosomal genes. Differences in response are not usually all or none but are concerned with quality and/or specificity of the antibody response.

The Ir-1 locus is closely linked to the H-2 gene in mice. Evidence indicates responsiveness to the various peptides (L-tyrosine and L-glutamic acid [T,G], L-phenylalanine and L-glutamic acid [P,G], and L-histidine and L-glutamic acid [H,G] built on multichain poly-DL-alanine) are under the control of different Ir-1 alleles (MCDEVITT, 1968). All of these polypeptides are antigenic in some strain of mice, but not necessarily in any one strain. They will not cross-immunize although antibody will cross react extensively. The Ir-1 gene functions at the T-cell level and controls cellular immune functions in the recognition of antigens, thus low responders are those who have reduced numbers of detectable precursor cells or cells with lower affinity for the specific immunogens. Low responder animals can, however, recognize these polypeptide antigenic determinants and produce large amounts of antibody when these hapten polypeptides are coupled with an immunogenic protein carrier. This shows one means of bypassing genetic defects (MCDEVITT, 1968).

The Ir-1 genes appear to have at least 2 or 3 separate loci. The Ir-IgA gene controls the immune response of mice to allotypic and idiotypic determinants on the IgA myeloma proteins derived from BALB/c mice. The Ir-IgG gene is linked to different H-2 specificities than the Ir-IgA and controls the immune response to γG (γ2a) of the same antigen (LIEBERMAN and HUMPHREY, 1971, 1972).

Various immunological responses have been shown to be non-H-2 linked. In immune responses to the (T,G) or (Phe-G)-Prol--L portion of multichain synthetic polypeptides, the response is controlled by the dominant, autosomal, Ir-3 gene (MOZES et al., 1973). The SJL mouse is the prototype for the high responders and the DBA/1 for the low responders. These responses are expressions of B cell activity and demonstrate antibodies can be made to 2 determinants on the same antigen under the control of 2 genes. Other non-H-2 linked gene controls of immune responses are reviewed by MCDEVITT and LANDY (1972), demonstrating dependence on the recognition of the antigenic determinant.

Antibody response to streptococcal polysaccharides differs dramatically with some strains, producing more to the group A than the group C carbohydrates, whereas in others, the reverse is true. These responses can give rise to a rather homogenous or a wide variation in the heterogeneity of the immune responses. B cell dependent responses to Salmonella lipopolysaccharides have also shown differences in mouse strain response and are dependent on recognition of the antigen (PAULI, 1972).

Having reviewed some of these various aspects of immunogenetic responses, one finds that new scientific discoveries are being made at a rate and volume beyond our ability to assimilate, evaluate, and make use of them as building blocks for rational progress. Unequal progress in the research prevents total integration of this knowledge into the understanding of host-parasite relationships. These studies with natural and synthetic antigens have demonstrated immunogenetic differences in responses which can not at this time be correlated with the viral and tumor specific membrane antigens. Recent advances in characterizing the amino acid sequences in the tumor virus (OROSZLAN et al., 1970; NOWINSKI et al., 1972) and the mouse myeloma protein (FRANCIS et al., 1974), the isolation of the viral envelope glycoproteins (KENNEL et

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al., 1973), and studies with carcinoembryonic antigens (TOMITA et al., 1974) will lead to a greater understanding of their immunogenetic potential. Each host responds to some of these antigens but probably not to all of the exposed antigenic configurations making up the tumor cell membranes and soluble antigens. The immunological reactions summarized in Table 2 for the various mouse strains in our carcinogenic studies amplify the subtle difference in their responses to various defined immunogens. Those differences in the capabilities of the host to mount an adequate response to produce a "killer" or "holding" effect influence the capabilities of these genotypic strains to allow "sneaking through" events to occur for the establishment of the initial carcinogenic event as a pathologic entity and also to influence the process of metastasis and the blocking phenomenon (HELLSTRÖM and HELLSTRÖM, 1970) that accompanies rapid growth and terminal events of the neoplastic process. The genetic capabilities of the host to respond may be one of the important factors governing the variation in latency period of tumor development in the animal models as demonstrated in Table 1.

We have elected to characterize the susceptibility of a mouse strain not only by the tumor incidence and latency but also by the carcinogenic index (IBALL, 1939), which equates susceptibility to latency and incidence of tumor induction. It is obvious that certain strains have longer latency periods than others yet produce comparable numbers of tumors. We cannot say at this time whether this represents an inability to respond to certain tumor antigens allowing "sneak through" events to occur or whether these inbred strains actually are capable of mounting a high level of response leading to the blocking phenomena and insuring rapid tumor growth. HALPERN (1973) developed high and low responder lines of Swiss mice to various unrelated antigens. These 2 lines were clearly separated for their humoral responsiveness but showed similar cell mediated reactions. When allogenic Sarcoma 180 implants were made, the high responders allowed the tumor to grow and 90% of the animals were killed, while in the low responders, all tumors regressed. The high responders synthesized high levels of antibody believed to have allowed the tumors to grow due to the blocking reaction, while the low responders produced only enough antibody to provide for effective cellular immunity. If the type C viral antigens play a significant role in chemical carcinogenesis, it may well be that of providing antigenic components in the tumor cell surface which may make them more antigenic as postulated by BARBIERI et al. (1971) and GREENBERGER and AARONSON (1973). On the other hand, a tolerance to these antigens may exist or these endogenous viral antigens may act rapidly to overload the system leading to the blocking phenomena. It is difficult to speculate regarding the wide divergence in antigenicity of chemically induced tumors. It could be assumed, however, that either the individual mice have a wide variation in capability to respond or that the antigenicity of the various chemically induced tumors varies significantly accounting for variations in the immunological response. Such variations in capability to respond would influence the time required for the blocking type phenomena to develop, thereby influencing the rate of tumor development on an individual animal basis (BARTLETT, 1972). The latter of the 2 hypotheses seems most likely since we are dealing with inbred strains, although we have noted a wide variation between individual mice in the mixed lymphocyte studies. We need to define molecular serology of antigens, antibodies, and antigen-antibody complexes in order to study the mechanisms of immune interaction in the mouse model system where the science of immunogenetics is well advanced. Various sensitive serological procedures, such as the radioimmune assay, are available for analyzing the specific antigenic components of the tumor antigens.

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Using such studies, we should be able to develop better diagnostic tools and, consequently, a knowledge of the potential usefulness of immunotherapy or immunological preventive procedures without producing an adverse stimulatory effect to those clones of transformed cells maintained in a "holding" state by host control mechanisms.

Other Genetic Controls of Neoplastic Developments

Many known genes have been associated with the occurrence of various forms of neoplasms; however, in many instances this relationship appears to have no connection with cancer development (HESTON, 1972). Early experiments in cancer research were concerned mainly with the inheritance of susceptibility to spontaneous neoplasia. The development of the inbred strains and the use of congenic strains have allowed for more specific genetic analysis of host susceptibility to carcinogenesis. In most cases, it has been shown that carcinogenesis is dependent on multiple genetic and environmental factors. We will review only a few of the genetic linkages reported to influence cancer development.

HESTON (1963) reported linkage between pulmonary tumors and 8 specific genes (hr, A^Y, vt, sh-2, wa-2, Fu, ah, and f), while LITTLE (1934), BITTNER (1945), and HESTON and DERINGER (1948) demonstrated linkage of mammary tumors with lethal yellow, brown, and agouti genes. STRONG (1945) linked gastric tumors with the brown gene while MACDOWELL (1945) and LAW (1952) demonstrated leukemia linked with dilute and flexed-tail genes. Although these associations have been made, their correlation with specific biochemical or physiological pathways has not been made. The H-2 histocompatibility loci has been associated with susceptibility or resistance to leukemia, the $H\text{-}2^k$ being considered susceptible while $H\text{-}2^b$ denotes resistance (LILLY, 1966). How the H-2 locus influences viral leukemia is not known but may represent influences discussed earlier in viral and immunological factor in the development of cancer.

MEIER et al. (1969) have described the genetic control of susceptibility or resistance to viral leukemogenesis by the hairless locus (hr). Hairless is an autosomal recessive mutation maintained in strain HRS/J. The incidence of leukemia is nearly 50% greater in the hairless (hr/hr) mouse than the haired mouse (hr/+) and occurs 6 months earlier. An N-tropic MuLV was isolated from both the hr/hr and hr/+ mice. The latency of neoplastic expression would appear to be related to an immunodeficiency factor rather than infectious virus expression (HEINIGER et al., 1974).

YAMAMOTO et al. (1973) propose that malignancy is induced by carcinogens by producing chromosomal changes resulting in a change in the balance between expression (E) and suppression (S) genes. E exists in normal cells but is neutralized by S, and malignancy occurs only when E increases or S decreases. Their studies with hamster cells injected with polyoma virus or treated with dimethylnitrosamine identified the location of the E and S chromosomes. These studies have been confirmed using Ara-C treatment of hamster cells (BENEDICT et al., 1974).

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Cells at Risk to Carcinogens and DNA Repair Synthesis

In addition to the previously discussed genetic factors that provide varying degrees of protection against the initial carcinogenic event, there is an additional factor we wish to discuss: the cells at risk to carcinogenesis. Many factors influence this population of cells, as dose of carcinogens, chronic exposure, site of exposure, aging processes, hormonal influences, stress factors that induce hormonal changes, diet that influences protein metabolism, and promoters such as dust, asbestos, physical, microbiological and chemical irritants, etc. The list is long and cannot be fully exploited here. If, however, one examines this list closely, it is found these factors have one thing in common. They all influence some function of DNA repair. PIERCE (1970) points out that tumors arise only in those tissues capable of mitotic activity. Chemical carcinogens transform only those cells which are entering mitosis which makes up only 0.03% to 0.13% of the body's cells at any one time (MEKLER, 1973). The cells at risk are few and far between unless there are intervening factors that stimulate unscheduled DNA repair. STICH and SAN (1973) indicate a link exists between the oncogenicity of a compound and its capability to provoke DNA repair synthesis. This, however, is not the entire picture, since cocarcinogenic effects do occur requiring both inducers and promoters. Cancer research has made use of this process for years in the form of croton oil in back painting carcinogenesis experiments. This has been carried over into lung carcinogenesis with the use of ferric oxide with BaP (SAFFIOTTI et al., 1968, 1972) and carbon dust or aluminum oxide with BaP (HENRY and KAUFMAN, 1973). STANTON and BLACKWELL (1961) and BLENKINSOPP (1968) stimulated repair and regeneration of pulmonary epithelium by pulmonary infarction. Chronic respiratory infections also promote regeneration of lung tissue and may play a role in carcinogenesis.

The various factors involving DNA repair have not been fully defined. HEINIGER et al. (1972) determined the overall DNA-turnover in 19 inbred strains and F1 hybrid mice. The range of DNA turnover observed suggested polygenic control (H-1, H-3, and H-4) of the steady state. The mouse strains with the shorter turnover time were C57BR/cdJ, DBA/2, SWR/J, and BALB/c. The intermediate turnover rate was represented by C57BL/10, C57BL/6, AKR, C57L, SJL, C58, and C3H/He, while DBA/1 has the longest turnover rate, which was almost 3 times that of C57BR/cdJ. The DNA turnover rate showed no correlation with spontaneous tumorigenesis.

Significant differences in ulcer formation in mice has been observed (LILLY and DURAN-REYNALS, personal communication; NEBERT et al., 1972). The form of host control appears to be primarily that of cells at risk. Much of the body receiving the greatest assault from carcinogenic agents is extracorporeal and has special defense mechanisms. The skin is made up of stratified layers of epithelial cells, with those involved in mitosis being the least exposed to toxic substances. In the respiratory and alimentary systems there is a high degree of vascularization and lymphatic involvement and secretatory activity which tends to produce rapid detoxification and also provides a high level of immunological protection decreasing the incidence of carcinogenic "sneak-through" events occurring. The ciliary action of the respiratory tract also provides for elimination of particulate irritants that induce cellular division.

In the experimental animal models, most chemically induced tumors have had their origin in cells other than the epithilial cells. However,

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in lung carcinogenesis, our primary concern is with the induction of squamous cell carcinomas. Alveoli are lined with squamous cells and are suspected to be the site of lung tumors. It is proposed that the use of ferric oxide or other particulate matter or chemicals that will induce mitotic activity increases the cells at risk and may in reality be only an exaggeration of what takes place in nature. Cigarette smoke carcinogenesis may be related to the cocarcinogenesis effects of weak carcinogens and particulate matter requiring extended chronic exposure. We have found that filtered cigarette smoke fails to induce AHH activity in the lungs of mice while unfiltered smoke induced for extended periods (KOURI et al., 1974). The particulate matter appears to play several roles, the trapping of AHH inducers and as promoters for weak carcinogen by increasing cellular proliferation. For these reasons it would appear the use of those agents that bring about unscheduled DNA synthesis are warranted in experimental models. Although we have considered primarily the influence of DNA synthesis on chemical carcinogenesis, this process is also important in triggering the hypothesized depression of repressors of tumor virus genome, protovirus, or oncogenes that may play an integrated role in the host as the etiological agents of cancer, be they viral, chemical or viral-chemical.

Summary and Application in Experimental Pulmonary Carcinogenesis Studies

The selection of animal models for cancer research must be based on the understanding and subsequent characterization of those host regulatory systems that define the differences between susceptible and resistant hosts. We have reviewed such host factors that appear to play decisive roles in carcinogenesis in the inbred mouse as an animal model. Differences in susceptibility to subcutaneous carcinogens with MCA, DMBA, and BaP have been demonstrated between various genotypically different strains of mice. Susceptibility to PAH carcinogens has been shown to be directly related to the inducibility of hepatic AHH, although differences in relative susceptibility to MCA, DMBA, and BaP were demonstrated in AHH inducible strains.

The host control of the type C RNA virus expression was reviewed. Although the frequency of occurrence of "spontaneous" neoplasia has been demonstrated to be related to infectious virus expression in mice, no direct influence of gs antigen or infectious virus expression on chemical carcinogenesis can be demonstrated. Various parameters of immunogenetics were discussed in relationship to the emergence of the initial transformed cells and the ultimate development of cancer. The immunosuppressive effects of carcinogens may play a role in the establishment of transformed cells and their growth. Differences in tumor latency are believed to be related to genetic differences in ability to recognize and respond to the various tumor cell antigens. The full impact of immunogenetics might be further understood when tumor cell antigens are more fully characterized.

We must consider not only the integrated host response but also the cells at risk to chemical carcinogens. Since DNA repair and mitotic activity may increase susceptibility to transformation, this aspect of susceptibility was discussed.

Based on our findings with subcutaneous chemical carcinogens in inbred mice, we have selected several strains for lung carcinogenesis studies. The effects of intratracheal inoculation of chemical carcinogens on AHH induction in the lungs (KOURI et al., these proceedings),

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and host immunocompetence (DEMOISE et al., these proceedings) will be correlated with respiratory tumor induction. Studies are also in progress using ferric oxide in combination with chemical carcinogens in hopes of increasing the cells at risk and inducing higher incidences of squamous cell carcinomas. The use of wax pellet carcinogen implants (STANTON and BLACKWELL, 1961) is also being evaluated as a means of inducing lung cancers in mice. By examining these various parameters and methods of tumor induction, we will evaluate the inbred mouse as a model system for lung carcinogens.

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Cell-Mediated Immunity after Intratracheal Exposure to 3-Methylcholanthrene, and its Relationship to Tumor Transplant Growth in C3H/f Mai Mice

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ABSTRACT

Immunological deficiencies have often been observed to occur in association with cancer although the exact nature of this relationship has not been fully characterized. The relative immunocompetence of an individual definitely plays a major role in the ultimate susceptibility or resistance to cancer. Numerous studies support the concept that cell-mediated immunity (CMI) is largely responsible for the body's defense against cancer. Our laboratory is currently interested in the levels of chemicals at which tumorigenesis occurs in various strains of mice and whether immunocompetence of the animals is affected.

In this investigation C3H/f Mai mice were intratracheally instilled 4 times at weekly intervals with 500 µg of 3-methylcholanthrene dissolved in a corn oil vehicle. These treatments caused 8% lethality in 30 days; whereas vehicle alone is nontoxic. Effects on CMI were determined 3 days after each treatment by measuring rates of DNA synthesis with ^{3}H-thymidine in allogeneic and spleen lymphocyte cultures. Spleen, thymus, and lung weight as well as blood leukocyte counts were measured. Syngeneic and allogeneic tumor transplants were performed on control and test mice to determine whether CMI data is biologically relevant to the process of tumor growth. The CMI and tissue responses were again evaluated 7, 14, and 28 days after tumor transplantation.

Preliminary data indicates that CMI, as reflected in spleen lymphocyte responses to phytohemagglutinin, pokeweed mitogen and allogeneic antigen, was suppressed during intratracheal instillations of 3-methylcholanthrene. This effect was most pronounced in response to pokeweed mitogen and persisted at least 2 weeks after exposures were discontinued. Lymphocyte cultures from mice that received tumor transplants indicate that the earlier CMI inhibition produced by carcinogenic exposure is not only cancelled but actually enhanced although only syngeneic transplants were successful. Again, it will be of interest to follow the kinetics of this effect in the host and compare it to the rate of tumor transplant growth.

A. Introduction

The intratracheal instillation of polycyclic hydrocarbons in hamsters, mice, and rats has served as a useful model for studies of respiratory carcinogenesis (SAFFIOTTI et al., 1968; NETTESHEIM and HAMMONS, 1971; SCHREIBER et al., 1972; SAFFIOTTI, 1969). Our laboratory is currently interested in the physiological effects of some of these chemical

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carcinogens, intratracheally instilled at concentrations known to induce respiratory malignancies. Evidence has accumulated during the past 15 years that clearly indicates the important role of host immunity in controlling the onset and progression of malignant diseases (MORTON, 1974). In this study as in others, we are characterizing some of the effects of chemical carcinogens on levels of host immunocompetence and their relationships to tumorigenesis.

B. Materials and Methods

I. Animals

Male C3H/f Mai mice 8 weeks old (Microbiological Associates, Walkersville, Maryland) and C57BL/6 Cum mice (Cumberland View Farms, Clinton, Tennessee) of similar age and sex were kept in disposable plastic cages containing corn cob bedding. They were given drinking water containing tetracycline (1 g/liter) and Purine Laboratory Chow ad libitum. A 12-hour lighting cycle was used.

II. Intratracheal Instillation of 3-Methylcholanthrene

C3H/f Mai mice were intratracheally instilled with 3-methylcholanthrene (MCA) according to the technique of HO and FURST (1973). Metofane (Pittman-Moore, New Jersey), an inhalation anesthetic, was used to anesthetize the mice. MCA (500 µg) was dissolved in 0.02 ml of corn oil (CO) and instilled in each of 100 test animals with a 19-gauge blunt needle attached to a Hamilton microliter syringe. A total of 4 such dosages were administered at 1 week intervals. Control mice (100) received only 0.02 ml CO.

III. Transplantation of Tumor Cells

Single-cell suspensions of syngeneic and allogeneic tumor cells from tissue cultures of a MCA induced C3H/f Mai tumor (passage 2) and a spontaneous BALB/c tumor (passage 2)(obtained from Dr. R. MADISON, Microbiological Associates) were diluted in Hanks' Balanced Salt Solution (1×10^7 cells/ml) for transplantation. Syngeneic and allogeneic tumor cells (1×10^6/0.1 ml) were inoculated into both MCA and CO exposed mice 2 days after the fourth, and final, intratracheal instillation. These were inoculated subcutaneously over the forehead for ease of palpation and measurement of growth.

IV. Culture Media, Mitogens, and Allogeneic Antigen

The culture media used was RPMI No. 1640 from Microbiological Associates supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, 100 µg/ml of streptomycin and 200 mM of L-glutamine. Phytohemagglutinin-M (PHA) and pokeweed (PW) mitogens purchased from Difco (Detroit, Michigan), and Gibco (Grand Island, New York), respectively, were reconstituted in sterile-distilled water and used at a final concentration of 1% v/v in culture media. Freshly prepared single-cell suspensions of spleen cells from C57BL/6 Cum mice were exposed to 4,000 R of X-irradiation and served as a source of allogeneic antigen.

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V. Spleen Lymphocyte Culture

The relative reactivities of thymus-derived (T) and bone marrow-derived (B) spleen lymphocytes were distinguished *in vitro* by their responses to mitogenic challenge with PHA and PW respectively (ANDERSSON et al., 1972). T-cell activity was further assessed in the mixed lymphocyte culture (MLC) reaction in response to stimulation by allogeneic antigen (C57BL/6 Cum spleen cells)(PLATE and MCKENZIE, 1973).

Single-cell suspensions of spleen lymphocytes obtained from 5 individual mice sacrificed at regular intervals after each intratracheal instillation of MCA (3 days post-exposure) and at 7, 14, and 21 days after transplantation of tumor cells were prepared in the same manner for all *in vitro* assays of cell-mediated immune (CMI) activity. Spleens from MCA and CO mice were removed, their capsules opened with scissors, and the cells teased out into chilled media. The suspension was allowed to settle briefly to permit removal of connective tissue fragments and large cell clumps. After 3 centrifugations (1,000 rpm for 10 min) and rinses, the single-cell suspension of spleen lymphocytes was adjusted to a density of 6×10^6 cells per ml of media. For mitogen stimulated cultures, spleen lymphocytes (6×10^5/1.2 ml) were pipetted in quadruplicate aliquots into wells of Falcon No. 3040 plastic microtiter plates. PHA and PW were then added to give a final concentration of 1% v/v in each well. Media alone was used for unstimulated controls.

The assay for MLV activity was also set-up with 6×10^5 spleen lymphocytes per 0.2 ml of media per well from MCA or CO mice. Syngeneic (C3H/f Mai) and allogeneic (C57BL/6 Cum), X-irradiated spleen lymphocytes (6×10^5 cells in 0.2 ml media) were used for antigenic stimulation.

Both mitogenic stimulated and mixed lymphocyte cultures of spleen cells were incubated at 37°C for 48 hours in a 5% CO_2 atmosphere. One micro Curie of ^{3}H-thymidine was then added to each culture and incubated an additional 18 hours. After a total of 66 hours incubation, the cultures were harvested and the contents of each well were transferred to DEAE Whatman No. 81 filter pads and allowed to dry. These filters were washed 4 times with 5% dibasic sodium phosphate, followed by 5 washes with distilled water, and again dried. Filters were transferred to vials containing 10 ml of Liquifluor (Beckman Instruments, Fullerton, California) and counted in a Beckman model LS 250 scintillation counter. Responses of T and B populations of spleen lymphocytes were expressed as the difference in counts per minute (ΔCPM) between the unstimulated and mitogen stimulated cultures. Similarly, in the MLC reaction, the response of T cells was expressed as the difference in counts per minute (ΔCPM) between syngeneic and allogeneic antigen stimulated cultures.

VI. Host Tissue Measurements

Animal weights were obtained on all mice at weekly intervals and at times when mice were sacrificed for spleen lymphocyte cultures. Pooled thymuses and individual lung and spleens were weighed. The total number of peripheral blood leukocytes was obtained using a Fisher Autocytometer II (Fisher Instruments, Pittsburgh, Pennsylvania).

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C. Results

I. Effects of MCA Exposure and Tumor Transplantation on Animal Weight

Animal weights of CO control and MCA test mice recorded after each intratracheal instillation and after transplantation of syngeneic and allogeneic tumor cells are shown in Fig. 1. After 4 exposures and 24 days on test, MCA-mice showed a 10% loss of weight compared to the CO controls. This effect persisted even after intratracheal instillations were discontinued, although by day 40 of the mice previously exposed to MCA did gain weight. It is interesting to observe that mice receiving syngeneic transplants (5 days after the last MCA treatment) unterwent a pronounced loss of weight through day 46, 20 days after tumors were transplanted. As presented later, syngeneic cells grew but allogeneic tumor cells did not.

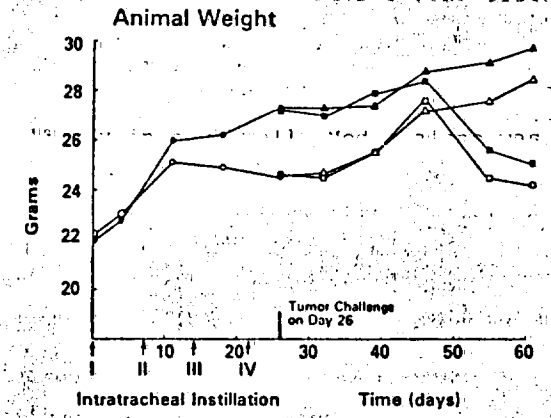

Fig. 1. Weight of mice in relation to intratracheal instillation of corn oil (solid circles) or 3-methylcholanthrene (open circles) and subsequent transplantation of syngeneic (squares) or allogeneic (triangles) tumor cells on day 26. Arrows indicate times of intratracheal instillation at 0, 7, 14, and 21 days.

II. Effects of MCA on Host Tissue Response

Table 1 shows the effects of MCA exposure on thymus, lung, and spleen weights and on peripheral blood leukocyte counts. All of these tissues were noticeably altered. Loss of thymus weight ranged from -38% after 1 exposure to -60% after 4, whereas lung weight increased as a function of MCA exposure. Spleen weights were depressed during MCA instillation and rebounded after they were discontinued. Leukocyte counts appeared to have increased as a function of MCA instillation.

III. Growth of Tumor Transplants

Syngeneic and allogeneic tumor cells were transplanted in CO control and MCA test mice, but only the syngeneic transplants grew into tumors, as indicated in Table 2. Tumors were first detected by palpation in both CO and MCA mice at 10 days after transplantation. It is interesting that tumors in MCA-exposed mice were significantly ($p < 0.01$) smaller than those in CO mice at 13 days and were also somewhat smaller at 28 days after transplantation.

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Table 1. Response of host tissues to 3-methylcholanthrene exposure[a]

Days on Test	No.[b] of Rx	ORGAN WEIGHT (grams) THYMUS C O	THYMUS MCA	THYMUS % Change	LUNG C O	LUNG MCA	LUNG % Change	SPLEEN C O	SPLEEN MCA	SPLEEN % Change	TOTAL CELLS (x 10^3) LEUKOCYTES C O	LEUKOCYTES MCA	LEUKOCYTES % Change
3	I	.047	.029	−38	.052	.069	+11	.065	.057	−12	3.2	2.9	− 9
10	II	.047	.028	−40	.228	.243	+ 7	.104	.088	−15	6.0	5.7	− 5
17	III	.055	.039	−29	.224	.250	+12	.068	.059	−13	5.0	6.0	+20
24	IV	.055	.022	−60	.214	.236	+10	.064	.061	− 5	4.0	7.0	+75
28	IV	.044	.027	−39	.186	.215	+16	.054	.070	+30	6.7	7.6	+13
35	IV	.033	.034	+ 3	.162	.191	+18	.064	.084	+31	6.6	8.0	+21

[a]Weights and cell totals represented as arithmetic mean of 10 mice

[b]Cumulative number of intratracheal instillations of 500 ug 3-Methylcholanthrene (MCA) in 0.02 ml corn oil (C O)

Table 2. Syngeneic and allogeneic tumor growth in 3-methylcholanthrene exposed mice

Tumor Type[a]	Treatment[b]	Tumor Size (cm) at Days After Transplantation 10	13	28	Tumored Mice No. Tested	(%)
Syngeneic	C O	palpable	0.94	1.91	26/26	(100)
	MCA	palpable	0.75	1.79	26/26	(100)
Allogeneic	C O	no tumor	0.00	0.00	0/26	(0)
	MCA	no tumor	0.00	0.00	0/26	(0)

[a]1×10^6 syngeneic (C3H/fMai) or allogeneic (BALB/c) tumor cells were injected subcutaneously in mice five days after the fourth and final intratracheal instillation

[b]Corn oil (C O) alone or 500 ug 3-Methylcholanthrene (MCA) dissolved in C O were administered intratracheally four times at weekly intervals before tumor transplantation

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IV. Cell-Mediated Immune Effects of MCA Exposure and Tumor Transplantation

The relative reactivities of T and B spleen lymphocytes were determined *in vitro* with spleens removed from mice both during the intratracheal instillation of MCA and 7, 14, and 21 days after transplantation of tumor cells. Spleen lymphocyte responses to PHA, a T-cell specific mitogen, are shown in Fig. 2. Although some depression of T-cell activity occurred during intratracheal instillation of MCA, it reached significant levels only after the second ($p < 0.02$) and fourth (p 0.01) exposures. This depressed response returned to normal within 3 days after instillations of MCA were discontinued. One may observe that CO instillation in itself lowers T-cell activity after the second and third exposures. Most striking in Fig. 2 was the noticeable enhancement of T-cell reactivity produced by both syngeneic and allogeneic tumor cell transplantation regardless of whether the host was previously exposed to MCA or not. T-cell immune activity also was measured in response to allogeneic antigen in mixed lymphocyte cultures, and these results are shown in Fig. 3. Levels of T-cell reactivity were not effected by MCA exposure, but again were noticeably enhanced by transplantation of syngeneic and allogeneic tumor cells.

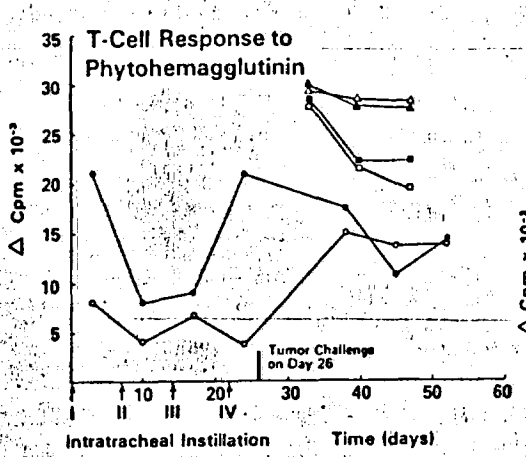

Fig. 2

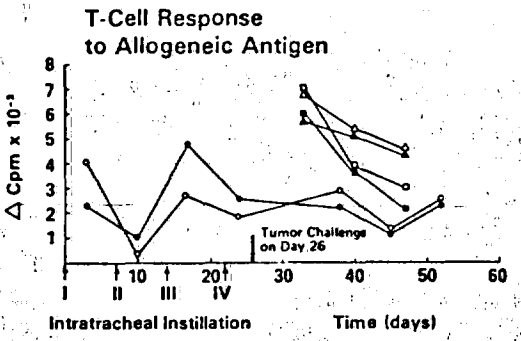

Fig. 3

Fig. 2. Response of spleen lymphocytes to mitogenic stimulation with phytohemagglutinin after intratracheal instillation of corn oil (solid circles) or 3-methylcholanthrene (open circles) and subsequent transplantation of syngeneic (squares) or allogeneic (triangles) tumor cells on day 26. Arrows indicate times of intratracheal instillation at 0, 7, 14, and 21 days

Fig. 3. Response of spleen lymphocytes in mixed lymphocyte culture to allogeneic antigen after intratracheal instillation of corn oil (solid circles) or 3-methylcholanthrene (open circles) and subsequent transplantation of syngeneic (squares) or allogeneic (triangles) tumor cells on day 26. Arrows indicate times of intratracheal instillation at 0, 7, 14, and 21 days

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Whereas T-cell spleen lymphocyte activity was only periodically effected during MCA exposure, bone marrow-derived, B-lymphocyte activity was significantly depressed as shown in Fig. 4. Pronounced levels of depression occurred after instillations at 0 ($p < 0.0005$), 7 ($p < 0.01$), 14 ($p < 0.025$), and 21 ($p < 0.025$) days and remained depressed for 2 weeks after exposures were discontinued. Both T- and B-cell activity were stimulated by syngeneic and allogeneic transplanted tumor cells and again this occurred independent of whether the host had previously been exposed to MCA or not.

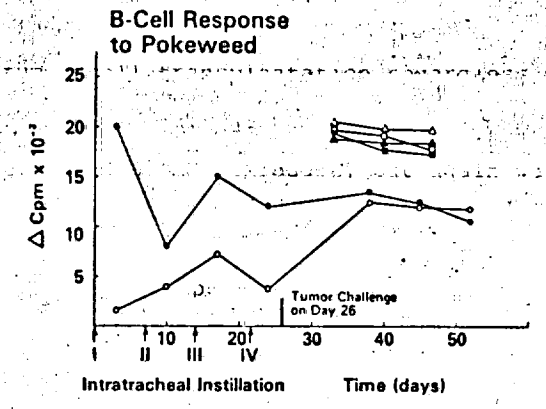

Fig. 4. Response of spleen lymphocytes to stimulation with pokeweed mitogen after intratracheal instillation of corn oil (solid circles) or 3-methylcholanthrene (open circles) and subsequent transplantation of syngeneic (squares) or allogeneic (triangles) tumor cells on day 26. Arrows indicate times of intratracheal instillation at 0, 7, 14, and 21 days

D. Discussion

Contrary to the expectation that MCA exposure might lower host immunocompetence to the point of allowing better growth of transplanted tumor cells, it instead exhibited a tumor-inhibitory effect, at least during the early stages of tumor growth. That transplanted syngeneic tumor cells did not grow as well in mice treated with MCA as in controls treated with CO might be related to the significantly depressed levels of B-cell activity incurred during the intratracheal instillation of MCA. This immunosuppressive effect might have been sufficient to influence at least the early stages of tumor growth. MCA is known to have an immunosuppressive property which impairs the function of B lymphocyte populations and thereby depresses the level of humoral antibody (BALL, 1970; STJERNSWÄRD, 1966; STUTMAN, 1969). Others have shown that antigens elicited on the surface of tumor cells can combine with circulating humoral antibodies to form antigen-antibody complexes effective in the prevention of tumor cell destruction by T-cell effector lymphocytes (BALDWIN et al., 1972, 1973; HELLSTRÖM et al., 1969). This being the case, lower humoral antibody levels in this study as indirectly suggested by the depressed B-cell activity in MCA exposed mice before tumor cell challenge would have permitted a more effective control of tumor growth. However, it is also possible that residual MCA in systemic circulation was simply cytotoxic to transplanted tumor cells and slightly inhibited their growth. Tumor inhibitory effects have been reported by others and have been compared to the deleterious action of x-ray, nitrogen mustard, methotrexate, and other agents on cell proliferation (HUGGINS and MCCARTHY, 1957; THOMPSON et al., 1960). It is difficult to separate the immunosuppressive and cytotoxic capacities

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of various agents (STUTMAN, 1973). Of course, with regard to carcinogenicity, it has already been demonstrated that repeated intratracheal instillation of MCA in mice eventually produces respiratory malignancies (HO and FURST, 1973b; NETTESHEIM and HAMMONS, 1971). To whatever degree mouse immunocompetence was altered in this study, it was not sufficient to overcome a strong histocompatibility barrier and permit growth of transplanted allogeneic tumor cells.

Cell-mediated immune activity as demonstrated by B- and T-cell responses to mitogenic and allogeneic antigen stimulation was clearly enhanced by tumor transplantation regardless of any effect of MCA exposure or tumor growth. The 10% loss of weight by mice exposed to MCA might have been expected to produce a weakened physiological state and a specific loss of immunocompetence. Increased weight of lungs in exposed animals probably was caused by a collection of fluids and infiltration of lymphocytes as an inflamatory response to irritation. The pronounced loss of weight by thymus tissues in response to MCA might well have affected alterations in levels of CMI. Peripheral leukocyte counts were elevated in response to MCA and further reflected chronic irritation of the respiratory tract. However, cell-mediated immune activity was clearly stimulated in response to tumor transplantation and appeared to be independent of any previous MCA induced changes in thymus, lung, spleen, or leukocyte host tissues.

This study suggests that pulmonary exposure to polycyclic hydrocarbons in mice provides a useful model for characterization of the underlying mechanisms of respiratory carcinogenesis and host immunocompetence.

E. Acknowledgements

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Cigarette Storage

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

March 4, 1975

To: The Scientific Advisory Board

Subject: Supportive Contract No. 20A - Microbiological Associates

The annual contract covers storage of cigarettes, purchased from the University of Kentucky, to be used in the inhalation and smoke fractionation studies of CTR contractors.

J.H.K.

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RENEWAL

AGREEMENT BETWEEN

MICROBIOLOGICAL ASSOCIATES

AND

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A.

This agreement covers services (to wit storage of test cigarettes under controlled conditions) by Microbiological Associates (MA) for The Council for Tobacco Research - U.S.A. (CTR-U.S.A.) and dated December 12, 1974.

It is agreed that total charges for these services will not exceed twenty-five thousand dollars ($25,000) for the fifteen-month period beginning April 1, 1975 and running through June 30, 1976. These charges do not include shipping costs which will be billed separately to the CTR-U.S.A. Payment to MA will be made on a monthly basis in equal installments within twenty (20) days after receipt of an invoice.

The services to be performed by Microbiological Associates include the following:

Provide sufficient refrigerated space to store up to fifteen million (15,000,000) test cigarettes at a constant 0°-3°C temperature.

Receive additional subject test cigarettes on a schedule to be agreed upon mutually by the Project Director (MA) and the Project Officer (CTR-U.S.A.) as space permits in the present facility.

Insure that the security and accountability provided conforms with the extant regulations of the Alcohol, Tobacco and Fire Arms Tax Division of the U.S. Treasury Department.

Provide an appropriate alarm system and physical security to protect these cigarettes which are the property of The Council for Tobacco Research - U.S.A.

Provide adequate insurance coverage against loss by fire and other disaster.

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Maintain a log-in and -out register of all receipts and issues. This record will be available for inspection by the Project Officer (CTR-U.S.A.) and/or the U.S. Treasury Tax Division on demand.

MICROBIOLOGICAL ASSOCIATES

BY: _____
Vincent Ruwet
Vice President, Contracts and Administration

ACCEPTED:

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A.

_____
W. T. Hoyt
Executive Director

_____
Date

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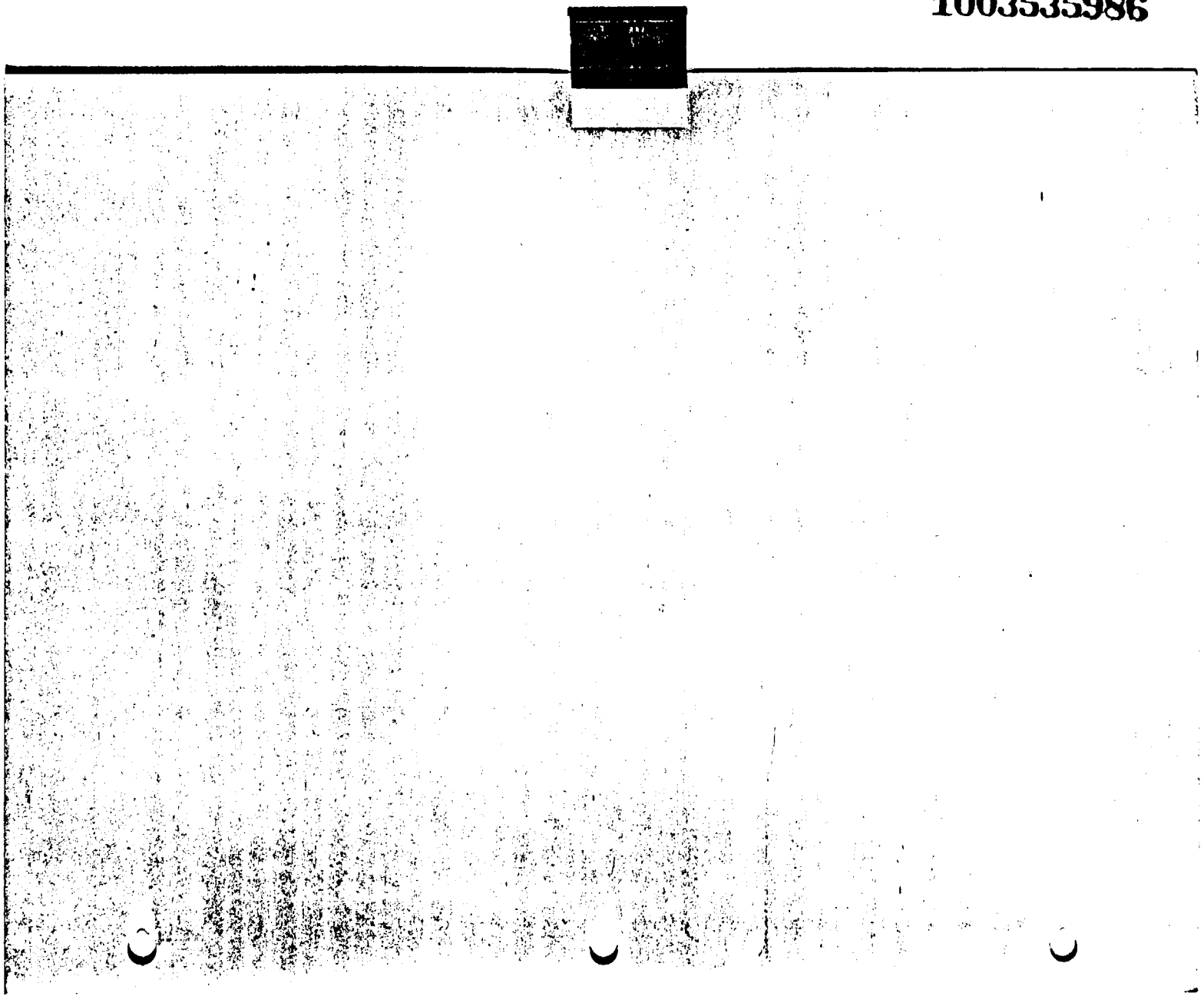

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Machine Evaluation
Dosimetry and Smoke
Fractionation

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

March 4, 1975

To: The Scientific Advisory Board of The Council for Tobacco Research - U.S.A., Inc.

Subject: Oak Ridge National Laboratory (CTR Contract No. C15B). "Smoking Machine Evaluation, Animal Dosimetry and Smoke Fractionation Progress"

Oak Ridge, under CTR Contract No. 15 has been developing test methods and technology, analyzing and evaluating smoking machines for a number of parameters of central concern to the smoke inhalation program. As you are aware, the smoking machines have improved greatly in function and sophistication in the last two-year period, significantly as a result of Dr. Guerin's and Dr. Stokely's interest in this program and their ready collaboration with other investigators in and out of CTR.

Currently, as can be seen by the attached progress report and projected outline of the next year, they are investigating the chemistry and particulate characteristics of smoke produced in the Walton and P&I Sem I Prototypes. Some studies of smoke produced in the Lorillard LACS II are also anticipated once it is operational.

A large body of data has been accumulated, and they have begun to write a series of papers on relevant topics. These include vertical versus horizontal smoking, positive versus negative puffing; effects on smoke chemistry, particle size distribution in various machines using various assay measures, description of methods to characterize intermittent and continuous smoke generator machines, effects of interfaced animals and chamber constituent depletion, and several general papers on smoke chemistry and machine characteristics. Collaboratively several papers are possible in conjunction with Dr. Whitmire at MA, Dr. Essman at Queens College, Dr. Greenspan and Mr. Florant at P&I.

For the next year some additional assistance will be required to optimally develope the P&I Semi II model. It would not have been possible to design and build the Sem I in such a short span (since last January) without such a collaborative effort among CTR contractors. Extensive dosimetry studies, collaboratively with Drs. Whitmire, Kouri, Demoise at MA, and taking advantage of the analytical techniques developed in this program, are underway to define localized tissue content of smoke particulate and gas phase constituents. These studies will study different mouse strains on varied multiple smoke exposure regimens.

Lastly, a small pilot project to initate subfractionation of smoke condensate at no great cost would be begun. This program would not be extensive but should investigate feasibility of adopting technology currently applied to petroleum fractionation to our problems. It should

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be clearly understood that this would be preliminary and not the extensive and more definitive colum chromotography, mass spectrographic program required to get answers rapidly in the future. Inasmuch as a preliminary program can give leads, it will be useful. Hopefully, some fractions could be tested for biological effects, such as IT and SC. cocarcinogenesis, AHH induction, S. typhimurium mutagenesis and specific tissue culture cell transformations.

J.H.K.

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OAK RIDGE NATIONAL LABORATORY
OPERATED BY
UNION CARBIDE CORPORATION
NUCLEAR DIVISION

POST OFFICE BOX X
OAK RIDGE, TENNESSEE 37830

FEB 20 1975

February 14, 1975

Dr. John Kreisher
Associate Scientific Director
Council for Tobacco Research-USA, Inc.
110 East 59th Street
New York, New York 10022

Dear John:

I have attached my estimate of the accomplishments expected within the framework of existing third year funding and additional studies I believe worthy of supplementary funding as of 6-1-75.

Please let me know which, if any, of these additional studies should be included in a formal proposal for supplementary work/funding. I will prepare the formal request as soon as I hear from you.

You will note from Jim's Interim Progress Report that significant progress has been made. Our only complication has come in the major modifications and repair required of the LACS II and P&I SEM I.

You will be pleased to hear that considerable progress has also been made in preparing open literature publications. Considering deadlines, they will not appear in the literature before our more detailed Progress Report preparatory to third year funding is forwarded to you.

I look forward to hearing from you.

Sincerely,

M. R. Guerin

MRG
Attachment

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APPENDIX I

PROJECTED CORE ACCOMPLISHMENTS*
(Without Supplementary Funding)

September 1975, 3 months into year 3

1. LACS II and P&I systems operational--at least well enough to test chemically.
2. Tests applied and results available as follows: mice present, ~10% smoke, 30/30 smoke/air cycle.

Measurement[1]	Purpose	Walton	P&I	LACS II
TC Monitor[2]	gp uniformity, age, purge	5 positions	7 positions	7 positions
Optical Monitor[2]	pm uniformity, age, purge	5 positions	7 positions	7 positions
GP Profile[3]	gp composition	1 position	1 position	1 position
TPM Profile[3]	pm composition	1 position	1 position	1 position
CO/CO_2	gp concentration, uptake/buildup	1 position	1 position	1 position
H_2O	humidity	1 position	1 position	1 position
Nicotine	pm concentration, nicotine uptake	1 position	1 position	1 position
Neophytadiene	pm concentration	1 position	1 position	1 position
Cost/Animal	cost/benefit	--	--	--
Mechanical	reliability, service requirements	--	--	--

[1] Relative to standard analytical smoke and nonrestrictive smoke.
[2] Continuous Measurement.
[3] Early and late (5 sec, 25 sec) in stand period.

December 1975, 6 months into year 3

1. Above, plus
2. Final report including following measurements: (a) DCBP or DTC dosimetry, (b) particle size growth and particle concentration.

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June 1976, 12 months into year 3

1. Above, plus
2. Identification of constituents extracted preferentially from gas phase and particulate phase (determine whether change in composition is significant).
3. Particle size growth and concentration as functions of puff parameters, mode (normal, reverse; free, restricted) of puffing.
4. Complete comparative scheme, criteria, measurements.

(2) Continuous measurement.

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APPENDIX II

"SUPPLEMENTARY" STUDIES

1. Special Measurements (BaP, HCN).

   a. BaP (Determination of trace constituent known to be carcinogenic). Method based on addition of carbon-14-labelled BaP to cigarette will be developed and applied to determine distribution of BaP in chamber(s). Determine whether measurements of nicotine, DCBP, DTC-C^{14}, and neophytadiene allow prediction of BaP concentration in unlabelled smoke and determine uptake by animals.

   b. HCN (Determination of known toxic and ciliatoxic constituent capable of reaction). Method based on standard colorimetric procedure or on nitrogen-selective gas chromatography will be developed and applied to determine concentration and distribution in chamber(s). Determine predictability of concentration from analytical measurements, loss to reaction if any, and uptake by animals.

2. Dosimetry by Depletion.

   It has been demonstrated (work for NCI, to be published) that the decrease in concentration of any constituent during the smoke stand period is a measure of the quantity of that constituent accepted by the animals, i.e., the dose of that constituent. Chamber depletion analysis thus allows determining the doses of actual smoke constituents and, being a non-destructive measurement, allows studying dose throughout a chronic exposure experiment.

   The proposed work:

   Using the mouse strain used in the Microbiological Associates chronic study, the depletions of nicotine, carbon monoxide, acetaldehyde, acrolein, isoprene, hydrogen cyanide (if 1-b is approved), catechol, glycerol, palmitic acid, phenol, and neophytadiene will be determined. Exposure conditions, including the test cigarette, will be the same as employed in the MICRO study.

   Above data will be studied to determine whether depletions of nicotine and carbon monoxide can be used to predict doses of other constituents and whether a constituent other than nicotine is a preferred particulate matter indicator for comparing exposure systems. Data will define MICRO exposure.

3. Multiple Inhalation Dosing

   Studies of the contribution of individual smoke constituents or smoke fractions to observed biological effects resulting from inhalation exposure require that techniques and instrumentation be available to allow multiple dosing. Studies of co-carcinogenic activity and individual susceptability also require or at least benefit from the availability of such instrumentation.

   Two approaches are possible. A pure compound, e.g., BaP, can be added to the cigarette to increase the concentration of that constituent in the smoke. Alternately, the compound or mixture can be metered into the chamber with the diluting air. The preferred approach depends on the experimental objectives--gases are best metered with the diluting air while some particulate components are best added to the cigarette.

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The proposed work:

A carbon-14 distribution system of the type described by Philip Morris will be constructed and a carbon-14 sensitive gas chromatographic system will be purchased to allow studies of the fate of smoke constituents (selected for interest in co-factor exposure) added to the cigarettes. Constituents found transferred to the mainstream smoke without decomposition will be documented as applicable to this mode of co-exposure. Sufficient quantities of the constituent to be added will be synthesized or purchased and purified to prepare spiked cigarettes for inhalation testing.

The Walton-Horizontal system will be studied for the practicality of four co-factor dosing approaches: (a) pre-admission of freshly generated gas phase of smoke, (b) addition of gases and low molecular weight organics to fresh air supply, (c) pre-admission of gases and low molecular weight organics, and (d) pre-admission of particulates, smoke and non-smoke related, using an aerosol generator. Studies will include the development of suitable methods and hardware to produce the co-factor dose and chemically determining the concentrations and stability of the co-factor during the exposure period.

Co-factors to be studied using either cigarette addition and/or independent introduction will include carbon monoxide (stable gas), nitric oxide (reactive gas), acetaldehyde (low molecular weight organic), BaP (particulate carcinogen), smoke gas phase, classic tumor promoters, and an inorganic/organic aerosol yet to be selected.

4. Special Services for Microbiological Associates.

A "complete" chemical characterization of the smoke produced from the cigarette used for biological studies at MICRO would provide the following advantages: (a) direct comparability with NCI studies of approximately 120 cigarettes, and (b) "complete" definition of the exposure medium.

The proposed work:

Using both standard analytical smoke generation and standard but non-restricted analytical smoke generation, the following constituents will be determined in duplicate in the smoke of one cigarette type: TPM, water, nicotine alkaloids, tar, acetaldehyde, acrolein, isoprene, formaldehyde, oxides of nitrogen, hydrogen cyanide, carbon monoxide, carbon dioxide, whole smoke pH, catechol, phenol, free fatty acids, titrimetric acids, glycerol, and neophytadiene. Condensate (assumed produced by Meloy Labs under separate CTR contract) will be analyzed for indole, skatole, titrimetric acids, pH, BaA, BaP, phenol and cresols.

5. Subfractionation of Condensate by Chromatography--Pilot Study.

Future work for CTR-USA at this Laboratory will increasingly address quantitative fractionation of condensed smokes in support of biological studies by other CTR-USA contractors. A promising option to the sometimes criticized "Stedman-type" fractionation is the "SARA" chromatographic fractionation approach developed specifically for petroleum-related samples.

The proposed work:

The "SARA" chromatographic separation system will be constructed, applied to a petroleum-related sample to insure proper operation, and applied to condensed cigarette smoke. Aliquots of a given condensate will be subjected to the separation at least three times and the fractions obtained will be analyzed to determine quantitative and qualitative reproducibility. It will be known

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whether the approach shows sufficient promise to warrent efforts to modify the systems for preparative scale operations.

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COST ESTIMATES

Supplementary Study	Man Year Ph.D	Man Year BS/MS	Man Year Tech.	Construction, Supplies, Equipment	Total Cost $/year	Completion Years
1. Special Measurements (BaP, HCN)	0.05	0.50	--	0.8 K	28.3 K	1.0
2. Dosimetry/Depletion	0.05	--	0.50	6.5 K	24.0 K	1.0
3. Multiple Dosing	1.00	1.00	--	25 K	125 K	2.0
4. Special Services, MICRO	0.05	--	0.50	0.5 K	18.0 K	1.0
5. SARA Fractionation	0.10	1.00	--	8.0 K	63 K	1.0
	1.25	2.50	1.00	40.8 K	258.3 K	

Cost Supplement	$258,300
Gas Chrom-Mass Spec	110,000
TOTAL COST	$368,300

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OAK RIDGE NATIONAL LABORATORY
OPERATED BY
UNION CARBIDE CORPORATION
NUCLEAR DIVISION

POST OFFICE BOX X
OAK RIDGE, TENNESSEE 37830

FEB 20 1975

February 14, 1975

Dr. John Kreisher
Associate Scientific Director
Council for Tobacco Research-USA, Inc.
110 East 59th Street
New York, New York 10022

Dear John:

I have attached four copies of an Interim Progress Summary describing our work for the period June 1974 to January 1975. We have attempted in this summary to give an overview of the work rather than specific details. I will be happy to furnish additional information on projects that are of special interest.

Our work on the exposure machines is progressing along nicely. The new smoke distribution valve has been installed on the SEM I and is working well. The SEM I is being interfaced with our gas chromatographs to study smoke composition. We received the new model of the Walton from P&I recently and are doing preliminary work on it now. The LACS II is still giving us problems, but our maintenance people think most of the difficulties have been resolved, and the machine should be operational soon. One preliminary dosimetry experiment with Carrie has been completed and the data is in the computer now. John Caton and I will visit Carrie next week to make final plans and last minute changes in the experimental protocol. We are quite excited about the new method for particle size measurement and have applied it to the Walton. Our initial data on the Walton shows there is rapid growth of the particle size during the exposure period. It is going to be interesting to see what happens to the particle size with the P&I and Lorillard systems.

Mike and I are busy now writing papers. We hope to have drafts of three papers to you within three or four weeks.

We look forward to seeing you in the near future.

Sincerely,

James R. Stokely
Analytical Chemistry Division

JRS/bwm
Attachment

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INTERIM PROGRESS SUMMARY

to

THE COUNCIL FOR TOBACCO RESEARCH--USA, INC.

on

CHARACTERIZATION OF ANIMAL EXPOSURE SYSTEMS

Prepared by
J. R. Stokely and M. R. Guerin
Analytical Chemistry Division
Oak Ridge National Laboratory

February 12, 1975

Analytical Chemistry Division

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INTRODUCTION

This memorandum summarizes recent progress on the project to characterize animal exposure devices. Progress for the period June 1974 to January 1975 is summarized. Only abbreviated discussions of experimental results, conclusions, and the current status of the project are presented. Detailed discussions are not included as they are more appropriately presented in open literature publications and an annual progress report will be submitted in June 1975.

CURRENT STATUS

The primary objectives of the project are to establish a battery of tests for evaluating devices for exposing animals to whole tobacco smoke, to apply these tests to devices of interest to the Council for Tobacco Research, and to formulate recommendations for elimination of discovered shortcomings in the devices. Initial efforts were directed at developing methodologies needed to test the devices. This effort is continuing at a reduced level. Emphasis at the present time is on applying available tests to evaluate the Walton-Horizontal, Lorillard LACS II, and Process and Instruments SEM I exposure systems. A number of shortcomings in these systems have been discovered to date. Means for eliminating shortcomings have been devised and implemented in some cases.

Studies on the Walton Horizontal smoking machine have been extensive and have included an in-depth evaluation of the operational features of the machine and an investigation of the chemical properties of smoke offered to animals with the device. Chemical studies include assessment of smoke uniformity in the exposure chamber, losses of smoke from the exposure chamber, and the effect of smoke aging and animal interaction on the concentration and composition of smoke offered to animals with the device. Initial studies on smoke dose received by mice have been completed and more extensive studies are to begin in the near future. Methodology for determining the particle size distribution of smokes in animal exposure systems was recently developed. Initial application of the methodology has been made to the Walton system. Completed studies on the system are being prepared for open literature publication.

Work on the Lorillard LACS II exposure system has been restricted to an extensive operational evaluation and preliminary study of the chemical properties of smoke offered with the system. Complete evaluation of the system has been delayed because of the number and severity of maintenance problems that have been encountered. Although the basic design of the mechanical and electronic components of the system appear adequate, insufficient care was taken in construction of elec-

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tronic circuits that control operation of the system. Continual failure of the electronic circuits has occurred, and a major effort has been required to locate and repair faulty circuits. The system is presently inoperative, and a number of electronic circuits are being reconstructed.

A prototype model of the Process & Instruments (P&I) SEM I exposure system was received during this report period and an extensive operational evaluation was made. Several shortcomings of the system were discovered and design changes were implemented. The design changes are being incorporated in a new model of the system which is presently under construction by P&I. Studies to define the chemical properties of smoke offered by the prototype system are currently underway.

ACTIVITIES FOR THE PERIOD JUNE 1974 TO JANUARY 1975

A. Operational Characterization and Design Improvements in the P&I SEM I System.

1. The electronic timing circuits which control operation of the smoke distribution valve did not permit accurate control of the exposure time. An improved timing circuit was devised and recommended to the manufacturer for inclusion in the final model of the system.

2. A temperature rise of from 65-70°C was found to occur in the vicinity of cigarettes during smoke generation. A temperature rise of this magnitude is not acceptable because of resulting changes in puff volume and because the chemical composition of the smoke may be altered. The temperature rise was reduced to 25-30°C by changing the design of the sidestream smoke hood and the air flow system. Further reduction in the temperature rise will require major design changes if chemical studies reveal that the smoke composition is affected.

3. The relative humidity of air in the dome area of the system decreases to less than 10% when several loads of cigarettes are smoked. Because of the possible effects of the low humidity on the burning rate of cigarettes, the chemical composition of the smoke, and deposition of smoke particulates in the animals, efforts are being made to humidify the dome supply air to 60% relative humidity at ambient temperatures. A device for injection of water into the air supply has been designed and built. The device is currently undergoing tests.

4. Fluctuations in dome pressure were encountered when the eject and loader assemblies operated. The fluctuations produced a change in the puff volume of from 3-5 ml. A ballast tank installed on the supply air system was found to reduce the pressure fluctuations to an insignificant level. The ballast tank will be incorporated into the final design of the system.

5. Operation and calibration of the SEM I is easy and uncomplicated. In about three months of heavy usage, no major mechanical breakdowns, component failures, or other maintenance problems have been encountered.

[illegible] number and severity of maintenance problems that have been [illegible] basic design of the mechanical and electronic

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6. Measurements of the sidestream smoke concentration in the dome area were made using nicotine and carbon monoxide as smoke indicators. Based on these measurements, contamination of the mainstream smoke with sidestream smoke is less than 0.5%.

7. The static burn rate of cigarettes in the machine under operating conditions was measured. This study showed that the burn rate is not affected by the recommended air flow rate through the dome (350 cfm).

8. A number of minor design changes based on the initial evaluation of the prototype model were made to the manufacturer in a letter of January 2, 1975.

B. Chemical Properties of Smoke from the P&I SEM I System.

1. Assessment of the quantity and chemical composition of smoke generated and delivered by the prototype system is currently being made. Initial studies deal with the total particulate matter, nicotine, tar, and water deliveries of cigarettes smoked on the system. High resolution gas chromatographic methods for smoke particulates and gas phase are being applied to investigate smoke composition.

A. [illegible] measurements in the P&I SEM I System

C. Operational Studies on the Walton-Horizontal Smoking Machine.

1. Measurement of the static burn rates of cigarettes showed that the machine would not smoke three cigarettes as designed. Air venting from the puffing dome flows over the cigarettes and causes the static burn rate to increase so that the cigarettes are consumed with less than the standard number of puffs. A solenoid valve was installed in the puff air supply to eliminate the problem.

2. The procedure for calibration of puff volumes with the machine has been simplified. Studies showed that the puff volume is not significantly affected by the resistance of the cigarette, eliminating the need for a separate calibration with each type cigarette smoked on the machine.

D. Chemical and Physical Properties of Smoke Presented to Animals with the Walton-Horizontal Smoking Machine.

1. The possibility of changes in smoke composition as the smoke stands in the exposure chamber was investigated using two high-resolution gas chromatographic profiling methods. These methods permit visualization of approximately 250 of the major organic components in the gas and particulate fractions of smoke. Profiles for smoke aged for 30 seconds were almost identical to fresh smoke indicating that very little or no compositional changes are occurring in the Walton exposure chamber during exposures up to 30 seconds.

2. Analyses were made for nicotine, nitric oxide, and neophytadiene in the Walton exposure chamber to determine the effect of aging on these important smoke components. Only with nitric oxide was aging shown to produce a change in the component concentration. With nitric oxide, the concentration decrease due to reaction with oxygen was very small--the concentration decreased less than 10% in a 30 second exposure.

3. The high resolution chromatographic profiling methods were used to establish the effect of animal interaction with smoke in the Walton exposure chamber. Appreciable interaction was found. Twenty mice cause a

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general overall decrease in the gas and particulate phase concentration. In addition, several components in the gas phase are affected more than other components resulting in compositional changes. During a 30 second exposure, the overall organic gas phase concentration decreases by nearly a factor of two. One peak in the gas phase profile almost completely disappears and others are affected to a lesser extent. *Animal interaction with smoke in the Walton exposure chamber is potentially a serious problem* and should be taken into account in using the system for biological studies.

4. Analysis of nitric oxide in the Walton exposure chamber during exposures of 20 mice showed that the mice remove nitric oxide from the chamber at an appreciable rate and therefore deplete the chamber of this important smoke constituent. *The nitric oxide concentration is depleted by approximately a factor of two during a 30 second exposure of 20 mice.*

5. Application of newly developed methodology for particle size measurements have been made to determine the effect of smoke aging on the particle size of the smoke aerosol in the Walton exposure chamber. *Initial results show* that the particle size is increasing rapidly during a 30 second exposure; *the particle size approximately doubles during a 30 second exposure.* Studies on the effect of animals on the particle size are currently underway.

6. Comparisons were made of the composition of smoke generated by the Walton smoking machine and the Phipps and Bird analytical smoking machine. The high resolution chromatographic profiling methods were used for this comparison. This study showed that *smoke generated by the Walton duplicates smoke generated under standard smoking conditions.* They also suggest that *positive puffing has very little effect on smoke composition.*

7. *Continual use of the Walton machine* without cleaning *results in build-up of smoke particulates on the internal surfaces of the exposure chamber.* Studies were made to establish how frequently the exposure chamber should be cleaned to avoid exposing animals to the gas phase constituents in equilibrium with the particulates deposited on the walls of the chamber. Analysis of the gas phase from the exposure chamber for organic constituents showed very low levels of smoke components after 40 cigarettes had been smoked on the machine. *Once-a-day cleaning of the exposure chamber is sufficient* to prevent exposure to components in equilibrium with deposited particulates.

8. Initial dosimetry experiments with mice were performed during the first contract year on the Walton smoking machine. Extensive studies have been planned which will be a collaborative effort of this laboratory and Microbiological Associates. This work will begin with final approval from CTR.

E. Maintenance Problems with the Lorillard LACS II Exposure System.

1. Numerous maintenance problems have arisen in attempting to perform an evaluation of the Lorillard LACS II system. *The main cause for the problems is poor construction of electronic control circuits* in the instrument. During this report period, over 25 faulty solder joints have been repaired, and approximately 20 integrated circuits and 17 transistors have failed and have been replaced. The entire electronic circuits of the system are currently being checked and some circuits are being rebuilt. Thorough evaluation of the system is not possible until the electronic circuits are operating properly.

5. [illegible] were used to es-
[illegible] in the Walton expo-

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2. The quad-valve assembly on the system is inoperative. Lorillard Research has been informed of the mechanical failure of the valve, and is presently redesigning a new valve.

F. Preliminary Studies of Smoke Delivered by the Lorillard LACS II.

1. Measurements were made on the age of the smoke delivered by the LACS II using a thermal conductivity monitor developed during contract year one. These measurements showed that the smoke is less than 5 seconds old when it reaches the animal exposure unit (all of the smoke is less than 5 sec. old and 50% of the smoke is less than 2.5 seconds old).

2. Studies on the quantity of smoke delivered by the LACS II show that the smoke is offered to all animals at the same concentration. These studies were made using nicotine as a monitor of smoke concentration.

3. Losses of smoke particulates in connecting tubing used with the LACS II are very small and do not present a problem. Losses in the quad and shuttle valves have not yet been established.

4. The temperature in the vicinity of cigarettes smoked on the LACS II has been measured. It was found that the maximum temperature is 50°C which may have an effect on the chemical composition of smoke generated by the system.

G. Methodological Development and Exposure Concepts

1. A method has been developed for measurement of the particle size distribution of smoke particulates in exposure systems. The method involves encapsulation of smoke particles in a polymer bead and examination by scanning electron microscopy. Initial application of the method has been made to study smoke aging in the Walton Horizontal smoking machine.

2. Exploratory studies have been made on application of Fourier transform infrared spectroscopy to study compositional changes of smoke in animal exposure systems. Present indications are that the technique can be used to assess the possibility of very rapid chemical changes which occur within the first few seconds after smoke is generated. Techniques for detecting very rapid chemical changes are not now available.

3. A monitor for continuous measurement of the smoke particulate concentration in exposure systems is under development. Both laser light scattering and light absorption are being considered. A device is needed for particulate monitoring to establish smoke uniformity and concentration.

4. In smoke exposure systems, different smoke generation methods are used. Studies are underway to establish if and how the smoke generation method affects the delivery of the cigarette or the composition of the smoke. The following smoke generation variables are under study: horizontal vs vertical cigarette placement, restrictive vs free smoking, and positive vs negative puffing. Results obtained to date suggest that small but discernable differences in cigarette delivery result from the different smoke generation methods, but that compositional differences in the smoke are not evident.

[illegible] systems are possible until the electronic circuits are operating properly.

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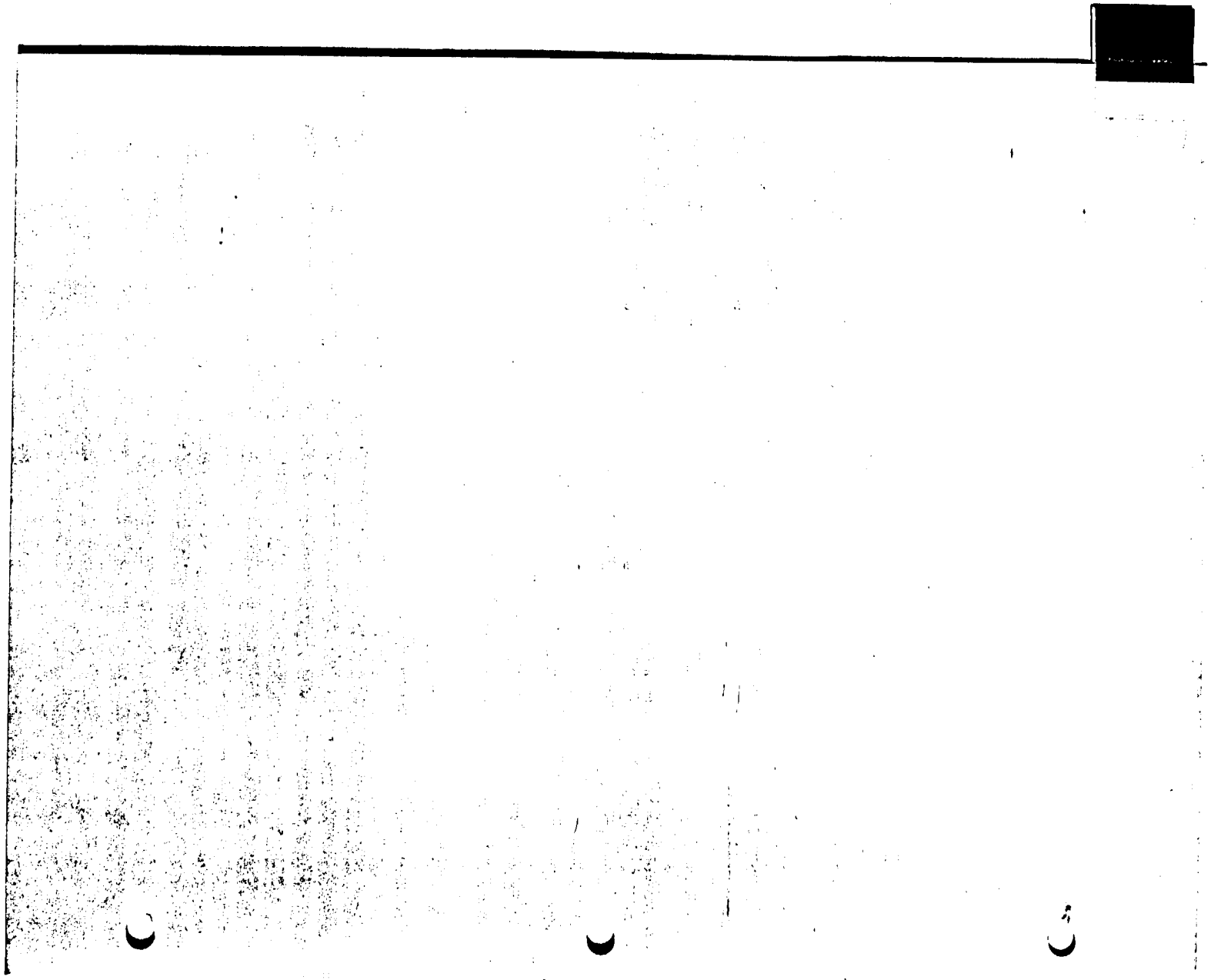

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Immunological Competence
and Chemical
Carcinogenesis

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Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

The Council for Tobacco Research - U.S.A., Inc.

110 East 59th Street
New York, N.Y. 10022
(212) 421-8885

FEB 3 1975

Contract
Application for Renewal of ~~Research~~ Grant (#1168)

1. Principal Investigator: Date: February 1, 1975

Richard A. Lerner, M.D., Member

2. Institution and Address:

Scripps Clinic and Research Foundation
476 Prospect Street
La Jolla, California 92037

3. Department where research will be done:

Department of Immunopathology

4. Short Title of Study:

Immunologic Competence and Chemical Carcinogenesis

5. Proposed renewal date:

July 1, 1975

6. How results to date have changed earlier specific research aims:

The specific research aim of this study continues to be the elucidation of the role played by the host immune mechanisms during chemical carcinogenesis.

In Phase I of the study we selected the best assay of immunocompetence. We then utilized this assay to establish the immunocompetence of both sexes of six strains of mice to five different antigens.

Phase II recently has been completed. It had previously been demonstrated that only strains which are so-called aryl hydrocarbon hydroxylase (AHH) inducible are susceptible to intratracheal (IT) chemical carcinogenesis by 3-methylcholanthrene (MCA). Essentially, we found that in these susceptible strains the IT administration of this carcinogen results in profound systemic immunosuppression. Phase III of the study will determine the importance of this immunosuppression in the development of lung tumors.

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7. How results to date have changed earlier working hypothesis:

There has been no change in earlier working hypothesis.

8. Any additional facilities now required?

No.

9. Any changes in personnel?

No.

10. Append outline of experimental protocol for ensuing year.

Our results from Phase II of this research demonstrated that in certain strains of mice 500 µg MCA is immunosuppressive when given IT. This only occurs in strains of mice which are susceptible to induction of lung tumors by IT MCA.

The next series of experiments will attempt to dissect chemical carcinogenesis from immunosuppression in this system. This is important because the approach to the control of lung tumor induction would differ considerably depending upon whether the immunosuppression we observed was the permissive factor permitting tumor growth; or was merely a biologic event having no etiologic import.

Since C_3H strain mice are sensitive to induction of lung tumors with MCA administered IT and also exhibit significant immunosuppression, we will utilize mice of this strain exclusively. The first experiment will be directed towards finding a dose of carcinogen that is sufficient to induce lung tumors but does not cause immunosuppression.

Mice will be given intratracheally either saline, gelatin, 9.38 µg, 18.75 µg, 75 µg or 300 µg of MCA at Microbiological Associates, Inc. (MAI). Mice are coded as to dosage of carcinogen or control by marking specific toes, and the assays are performed at Scripps without knowledge of dosage of carcinogen administered (i.e., blind). They will then be shipped from MAI to Scripps Clinic and Research Foundation and six days after intratracheal inoculation, mice will be immunized with either goat erythrocytes or saline. Ten days later, the mice will be re-immunized, and either three, five, or seven days thereafter, the number of cells in each spleen secreting antibodies to goat erythrocytes will be assayed by utilizing the Jerne plaque assay. This will be performed on a random integrated schedule so that control and experimental animals are carefully co-mingled each day. The calendar for this study is attached. Each manipulation noted on the calendar involves fifty individual mice.

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Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

This study will be completed by mid-year. The code will be broken and the data analyzed. We will then select a low dose of MCA, below the immunosuppressive threshold, and examine the immune response after multiple exposures to this low dosage.

Longer range plans are directed towards defining the role played by immunosuppression in permitting tumor development after chronic low dose exposure to chemical carcinogens and/or smoke.

11. List publications or papers in press resulting from this or closely related work.

Manuscript in preparation.

12. Summary progress report:

During the past year the second phase of this study was completed. This study was designed to determine whether or not doses of MCA sufficient to cause lung tumors when given IT also caused immunosuppression. Briefly, we found that in strains of mice that are sensitive to MCA carcinogenesis, systemic immunosuppression is observed after MCA is administered. Resistant strains of mice fail to exhibit systemic immunosuppression after MCA administration.

It has previously been shown that production of the aryl hydrocarbon hydroxylase (AHH) family of enzymes is induced by MCA, as well as other polycyclic aromatic hydrocarbons (PAH) in some strains of mice but not in others. The PAH are the substrate of these enzymes. MCA is only carcinogenic in the strains of mice in which it induces the AHH enzymes, and it is also only immunosuppressive in these strains of mice. Strains of mice that cannot catabolize MCA are not immunosuppressed by it, and do not develop tumors. This very important finding links immune competence to chemical carcinogenesis.

The following protocol was followed to arrive at the above conclusion. Goat erythrocytes (GRBC), with a mosaic of antigens on the surface, were selected as the test antigen. Three strains of mice were used (C_3H, $DBA/_2$, $C_{57}\ Bl/_6$); two are AHH inducible; one is not. Three different histocompatibility types (H-2) are represented. Either MCA in gelatin, gelatin alone, or saline was administered IT. Six days later, mice were immunized with GRBC, and ten days after that were re-immunized. Individual assays to quantitate the number of cells making antibody to GRBC were performed 3, 5, 7 or 9 days after the secondary immunization. This kinetic approach eliminated the possibility that a delay in peak response induced by MCA would be mistaken for true suppression. For each animal immunized with GRBC, an identical animal was immunized with saline; this allowed us to detect so-called "natural" immunity. Since we studied the secondary response to an antigen (two immunizations) a normal response was conclusive evidence that both thymus dependent and bone marrow dependent lymphoid populations were functional in these mice.

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Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

The results are presented on attached pages in tabular and graphic format. This study very clearly demonstrated significant immunosuppression lasting at least 19 days after IT administration of MCA in strains of mice that develop lung tumors after MCA is given IT (C_3H and C_{57} $Bl/_6$) and are inducible for the AHH enzyme system. In $DBA/_2$ mice, which are resistant to the carcinogenic effects of MCA and are AHH non-inducible, there is no immunosuppression observed. This is a very exciting result, as it presents us with an opportunity to dissect the role of immunosuppression in development of lung tumors after chemical carcinogens are administered.

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Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

APPENDED CALENDARS

FOR WORK TO BE PERFORMED

AS DESCRIBED IN SECTION 10

100353601O

Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

JANUARY 1975

MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY
		1	2	3
6	7	8	9	10
13	14	15	16	17
20	21	22	23	24
27	28 Inoculation of MCA at Microbiological Associates, Inc. and ship to Scripps	29	30	31

1003536014

Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

FEBRUARY 1975

MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY
3 1° injection of mice treated 28 Jan	4	5 Inoculation of MCA at MAI and ship to Scripps	6	7
10	11 1° injection of 5 Feb mice Inoculation of MCA at MAI and ship to Scripps	12	13 2° injection of 5 Feb mice	14
17 1° injection of 11 Feb mice	18 plaque assay of 28 Jan mice	19 Inoculation of MCA at MAI and ship to Scripps	20	21 2° injection of 5 Feb mice
24 plaque assay of 28 Jan mice	25 1° injection of 19 Feb mice inoculation of MCA at MAI and ship to Scripps	26	27 2° injection of 11 Feb mice	28

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Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

MARCH 1975

MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY
3 1° injection of 25 Feb mice	4 Inoculation of MCA at MAI and ship to Scripps	5	6 plaque assay of 11 Feb mice	7 2° injection of 19 Feb mice
10 plaque assay of 19 Feb mice 1° injection of 4 March mice	11	12 inoculation of MCA at MAI and ship to Scripps	13 2° injection of 25 Feb mice	14
17	18 plaque assay of 25 Feb mice 1° injection of 12 March mice inoculation of MCA at MAI and ship to Scripps	19	20 2° injection of 4 March mice	21
24 1° injection of 18 March mice	25 inoculation of MCA at MAI and ship to Scripps	26	27 plaque assay of 4 March mice	28 2° injection of 12 March mice
31 plaque assay of 12 March mice 1° injection of 25 March mice				

10035360 13

Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

APRIL 1975

MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY
	1	2 inoculation of MCA at MAI and ship to Scripps	3 2° injection of 18 March mice	4
7	8 plaque assay of 18 March mice 1° injection of 2 April mice inoculation of MCA at MAI and ship to Scripps	9	10 2° injection of 25 March mice	11
14 1° injection of 8 April mice	15 inoculation of MCA at MAI and ship to Scripps	16	17 plaque assay of 25 March mice	18 2° injection of 2 April mice
21 plaque assay of 2 April mice 1° injection of 15 April mice	22	23 inoculation of MCA at MAI and ship to Scripps	24 2° injection of 8 April mice	25
28	29 plaque assay of 8 April mice 1° injection of 23 April mice inoculation of MCA at MAI and ship to Scripps	30		

1003536014

Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

MAY 1975

MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY
			1 2° injection of 15 April mice	2
5 1° injection of 29 April mice	6 inoculation of MCA at MAI and ship to Scripps	7	8 plaque assay of 15 April mice	9 2° injection of 23 April mice
12 plaque assay of 23 April mice 1° injection of 6 May mice	13	14	15 2° injection of 29 April mice	16
19	20 plaque assay of 29 April mice	21	22 2° injection of 6 May mice	23
26	27	28	29 plaque assay of 6 May mice	30

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Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

GRAPHIC REPRESENTATION OF DATA

OBTAINED FROM STUDY OF IMMUNE COMPETENCE

IN MCA TREATED AND CONTROL MICE

A. $C_3H/_F$ MAI Mice

B. C_{57} $Bl/_6$ Mice

C. $DBA/_2$ -J Mice

D. Composite of All Data

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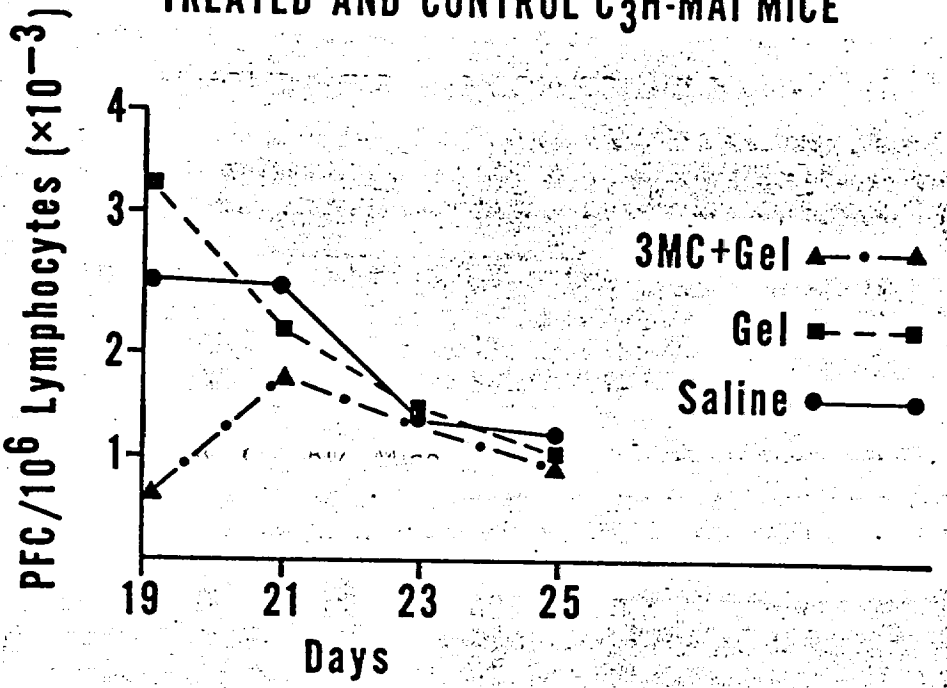
Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

ANTI-GOAT ERYTHROCYTE PLAQUE FORMING CELLS (INDIRECT) IN SPLEENS OF 3-METHYL CHOLANTHRENE TREATED AND CONTROL C_3H-MAI MICE

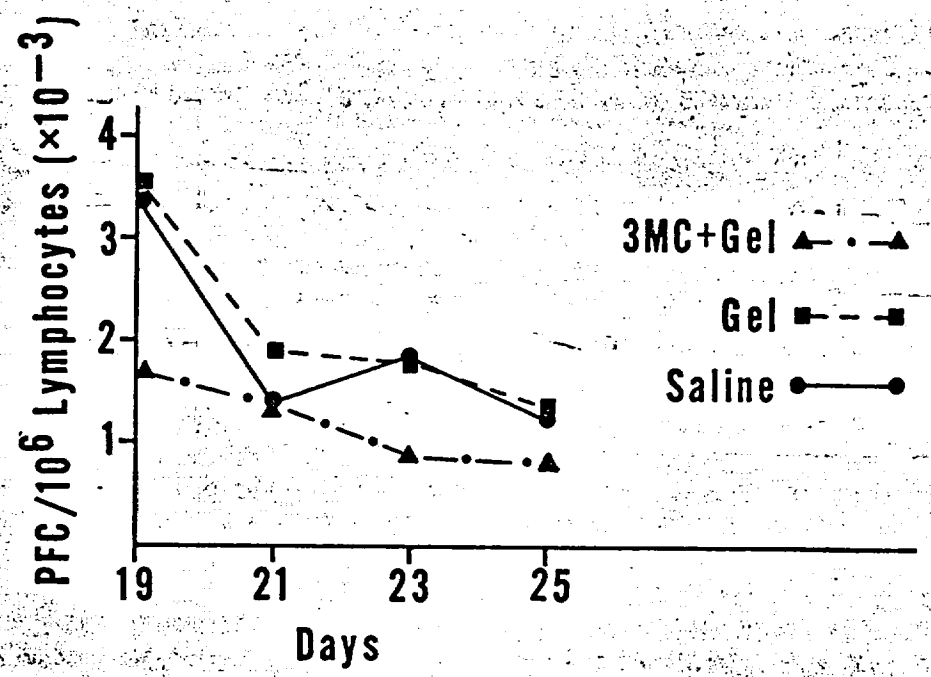


GRAPHIC REPRESENTATION - A

1003536017

Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

ANTI-GOAT ERYTHROCYTE PLAQUE FORMING CELLS (INDIRECT) IN SPLEENS OF 3-METHYL CHOLANTHRENE TREATED AND CONTROL C_{57}-BL/$_6$-Cum MICE

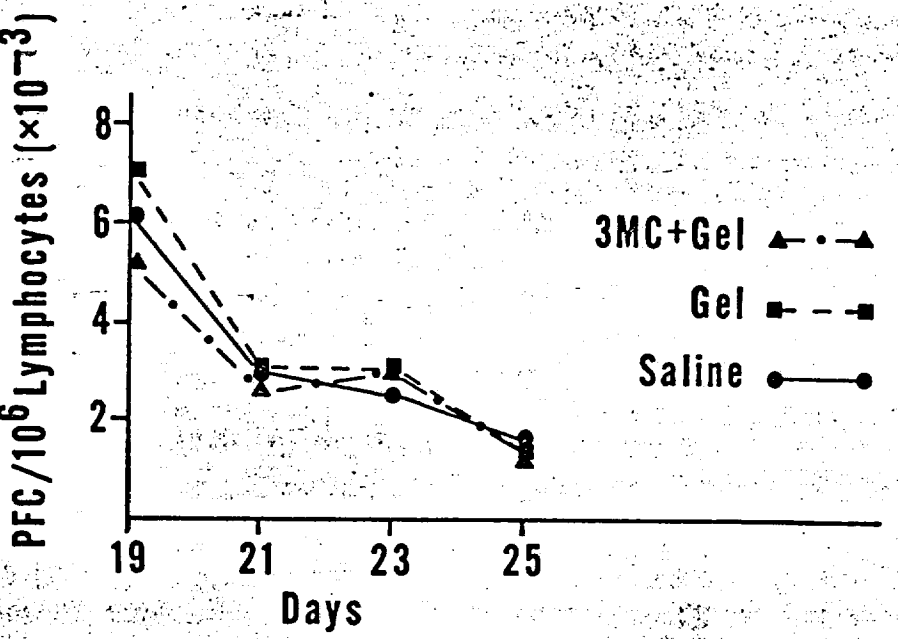


GRAPHIC REPRESENTATION - B

100353618

Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

ANTI-GOAT ERTHROCYTE PLAQUE FORMING CELLS (INDIRECT) IN SPLEENS OF 3-METHYL CHOLANTHRENE TREATED AND CONTROL DBA/2-J MICE

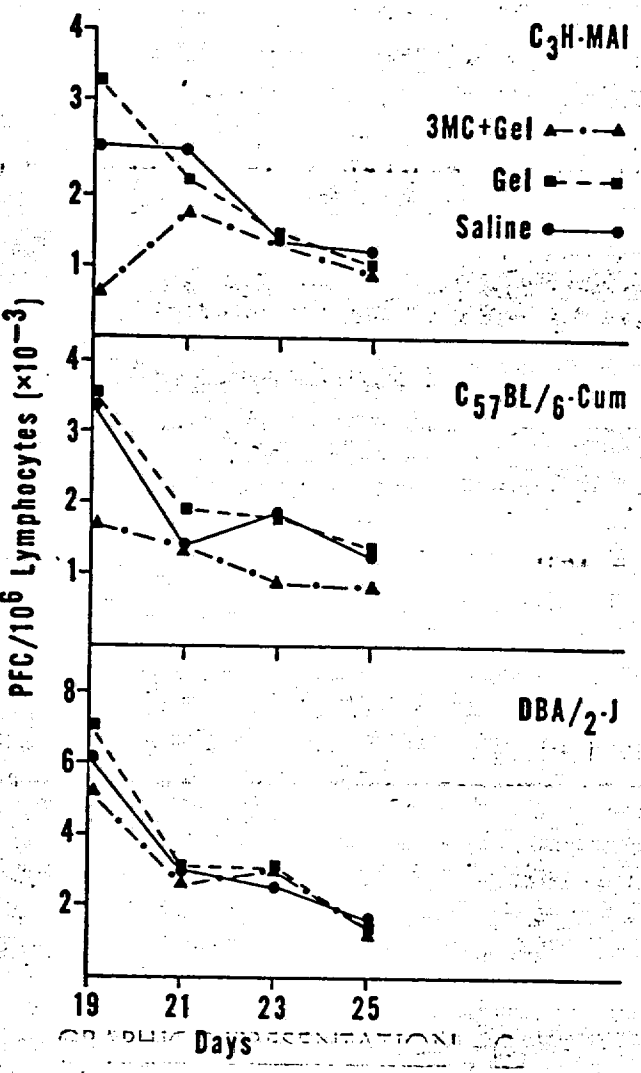


GRAPHIC REPRESENTATION - C

100353619

Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

ANTI-GOAT ERYTHROCYTE PLAQUE FORMING CELLS (INDIRECT) IN SPLEENS OF 3-METHYL CHOLANTHRENE TREATED AND CONTROL MICE



GRAPHIC REPRESENTATION - D

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Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

TABULAR REPRESENTATION OF DATA

OBTAINED FROM STUDY OF IMMUNE COMPETENCE

IN MCA TREATED AND CONTROL MICE

Each number represents 10 individual assays. Background, representing "natural" immunity, has been subtracted for each assay.

A. Indirect (IgG) Plaque Forming Cells Per 10^6 spleen lymphocytes.

B. Direct (IgM) Plaque Forming Cells Per 10^6 spleen lymphocytes.

100536021

Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

TABULAR REPRESENTATION - A

INDIRECT PFC/10^6 SPLEEN LYMPHOCYTES

		Day 13	Day 15	Day 17	Day 19
	N	344	1227	1470	1089
C_3H ♀	R	2828	2353	1553	1083
	L	2263	3157	1292	1284
	N	664	2218	899	552
C_3H ♂	R	3692	2143	1391	864
	L	2946	1944	1363	1095
	N	6310	3018	3479	1441
DBA ♀	R	6698	3681	3089	1685
	L	5157	3140	2727	1739
	N	3304	1679	2068	1302
DBA ♂	R	7152	2127	3043	1035
	L	6898	2242	2094	1423
	N	1629	1404	1165	900
$C_{57}Bl/_6$ ♀	R	3384	1890	2571	1242
	L	4364	1432	2186	1344
	N	1579	1238	555	640
$C_{57}Bl/_6$ ♂	R	3558	1785	1061	1472
	L	2422	1285	1493	1089

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Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

TABULAR REPRESENTATION - B

DIRECT PFC/10^6 SPLEEN LYMPHOCYTES

		Day 13	Day 15	Day 17	Day 19
C_3H ♀	N	198	82	215	81
	R	635	197	91	109
	L	519	172	243	171
C_3H ♂	N	366	124	77	59
	R	594	114	126	96
	L	563	216	79	102
DBA ♀	N	136	51	115	40
	R	109	76	104	77
	L	298	83	188	94
DBA ♂	N	93	41	27	43
	R	275	87	61	44
	L	423	447	58	170
$C_{57}Bl/_6$ ♀	N	638	139	78	80
	R	421	130	151	80
	L	734	101	127	71
$C_{57}Bl/_6$ ♂	N	511	115	56	109
	R	537	120	86	150
	L	575	186	124	110

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Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000

13. Budget for the coming year:

		% Time	Amount
A.	Salaries:		
	Professional:		
	Lerner, Richard A.	15%	-0-
	Levy, Richard L.	40%	9,280
	Technical:		
	Technicians (2)	100%	20,000
	Sub-Total for A		29,280
B.	Consumable Supplies:		
	Supplies 11,000		
	Animals 10,000		
	Sub-Total for B		21,000
C.	Other Expenses:		
	Travel and Shipping Costs 3,000		
	Part time services of secretary, animal caretakers, photographer, histology technician, EM technician, electronic repairman and machinist 4,000		
	Sub-Total for C		7,000
	Running Total of A+B+C		57,280
D.	Permanent Equipment:		
	None		
E.	Indirect Costs (15% of A+B+C)		8,592
	TOTAL REQUEST		65,872

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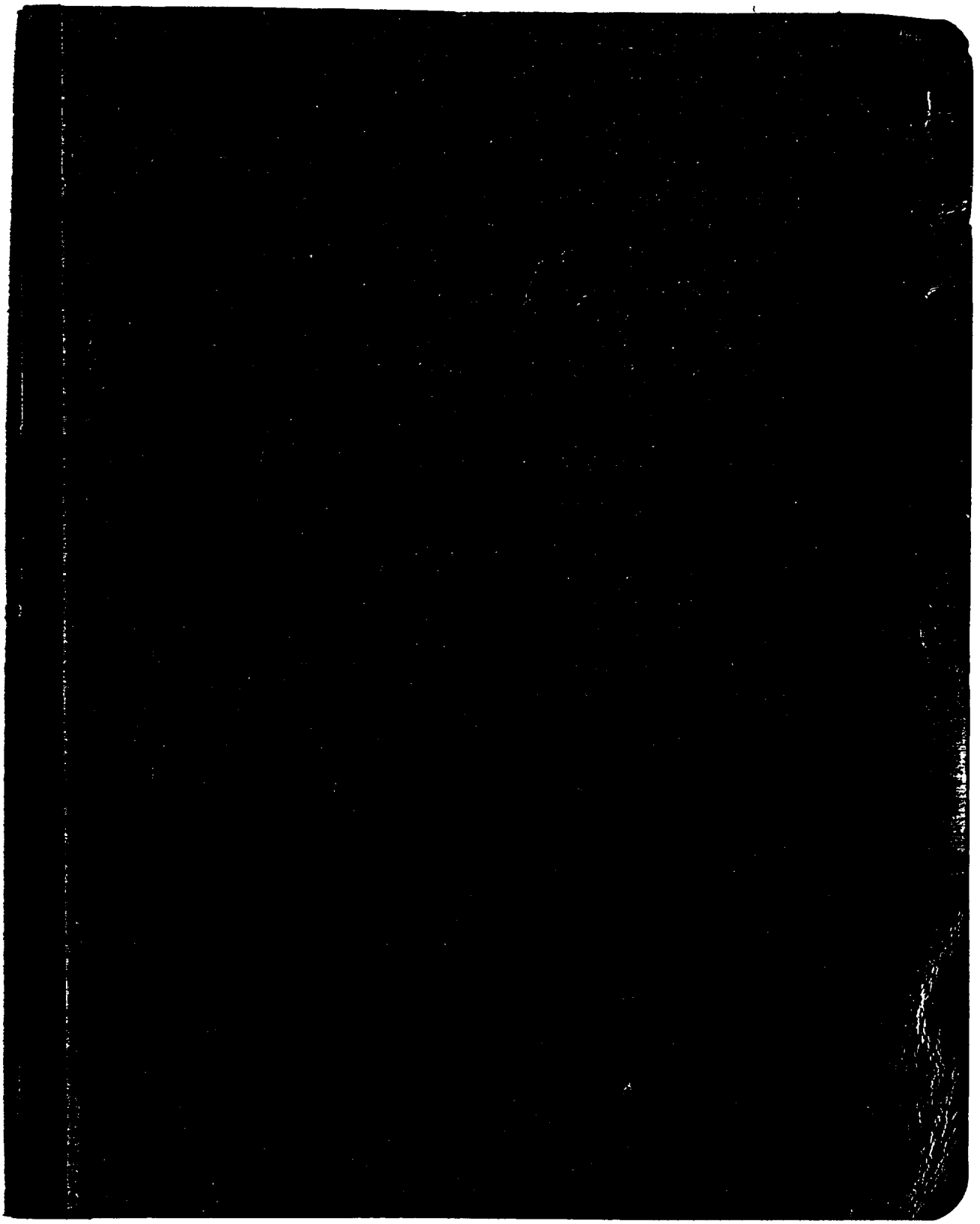
Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000



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Source: https://www.industrydocuments.ucsf.edu/docs/fgml0000